MODES OF ACTION OF ANTIMICROBIAL GARLIC DERIVATIVES

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ABSTRACT

Two commercial garlic products; a steam distillate of garlic (G.O) containing essential garlic oils AND a freeze-dried garlic powder (G.P) have been studied with respect to their antimicrobial modes of action with reference to gut environments.

Results obtained in this thesis indicate:-

1) A method of preparing G.P for use was developed and the allicin content (from alliin) determined by both pyruvate and ammonia assays to be approximately 11.00mg/g was shown to be comparable to published data.

2) Quantitative and qualitative methods for determining the sulphide and thiosulphinate content of G.O and G.P respectively, by gas chromatography (GC) analyses were performed. It was observed that G.P analysis (by conversion of allicin to vinyldithiins) provided an underestimate of the allicin content as compared to published data. For G.O seven sulphides were resolved, of these DADS, MATS and DATS contribute approximately 60% of the identified sulphide composition (70-80%).

3) G.O (and G.P) components were separated by high performance liquid chromatography (HPLC) and for G.O ten sulphides were resolved, of which eight were identified. Four of these sulphides (MATTS, DATTS, MAPS and DAPS) were not detected by GC analysis and in contrast MAD detected by GC was not identified by HPLC.

4) In addition HPLC analysis was used in conjunction with viability studies to monitor relative changes in G.O sulphide composition with respect to time in the presence of microbial cells.

5) Both G.O and G.P exhibited antimicrobial properties against all the bacteria screened. The antimicrobial effectiveness was shown to be dependent upon garlic product concentration and time of exposure but independent of whether the bacteria were growing or not.

6) MIC determinations indicated differences in bacterial sensitivity towards both garlic products with respect to both species and even strain. For G.P, MIC values ranged from 25-3.125mg/ml, suggesting a limited variation in bacterial sensitivity whereas a much larger MIC ranges of 5.5-0.01mg/ml were obtained for G.O. Certain pathogenic bacteria (*L. monocytogenes* and *Y. enterocolytica*) were shown to exhibit much greater sensitivity (MIC values of 0.02 and 0.17mg/ml respectively) towards G.O than other bacteria studied including normal microbial flora.

7) A decrease in G.O antimicrobial effectiveness with respect to time was observed during viability studies (not observed with G.P) and was partly attributed to the loss of antimicrobial components by volatilisation, interaction/association between the G.O sulphides and bacterial cells, and changes in chemical composition of the medium.

8) G.O was shown to be antimicrobially effective in a variety of media ranging from simple salt solutions, microbiological growth media (TSB and MRS), simulated intestinal fluids (simple and complex) to highly complex 'real' gut fluids (ileostomy effluents). In addition it was observed that the different chemical compositions of the various media can influence the antimicrobial effectiveness of G.O.

9) The presence of G.O (at specific concentrations) was shown to selectively reduce or eliminate the number of viable *L. monocytogenes* cells from a range of media including ileostomy fluid whilst allowing natural intestinal bacteria to proliferate. This was in addition to an antagonistic (biocidal) effect on *L. monocytogenes* of natural intestinal microflora and to some extent of non-cellular components of ileostomy fluid.

10) Individual dialkylsulphides (DMD, DMT, DAS and DADS), were shown to possess bacteriostatic and bacteriocidal activity. Comparison of the effectiveness with that of G.O indicated that alone these sulphides do not fully account for the high antimicrobial activity of G.O.

11) The effect of G.O on two enzymes (ADH and LDH) was tested. It was shown that lower G.O concentrations were required to inhibit ADH and LDH activity (0.0013 and 0.0027mg/ml respectively) as compared to the inhibition of cellular growth (0.01-5.5mg/ml), suggesting that cellular target sites are less sensitive to G.O than these two enzymes.

12) Progress in elucidation of the mode of antimicrobial action of G.O was provided by the following areas: A) G.O sulphides are antimicrobial components; B) action on enzymes AND C) SH-groups as target sites for garlic sulphides. Point C) comes from evidence of a variety of sources including; antimicrobial activity of sulphides, effect of cysteine and tryptone on G.O activity and effect of G.O and sulphides on SH-enzymes and glutathione studies.

13) Results obtained in this thesis indicated that achievement of microbiostatic and perhaps microbiocidal concentrations of garlic products intestinally may appear realistic. The future potential of the oral consumption of garlic to prevent or overcome food-borne infection is discussed and evaluated.

ACKNOWLEDGEMENTS

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I would just like to apologise for all the nasty smells I created with the garlic oil.

Special thanks go to my parents for their support, patience and understanding over the past 26 years.

"Using garlic as a proposed antimicrobial agent in this study, I might not have had a friend to turn to but at least I never caught a cold."

ABBREVIATIONS

The following abbreviations have been used in this thesis:

G.O	Steam Distilled Garlic Oil
DAS	Diallyl sulphide
DADS	Diallyl disulphide
DMD	Dimethyl disulphide
DMT	Dimethyl trisulphide
DATS	Diallyl trisulphide
DATTS	Diallyl tetrasulphide
MATS	Methyl allyl trisulphide
MATTS	Methyl allyl tetrasulphide
G.P	Freeze-Dried Garlic Powder
w/v	weight for volume
v/v	volume for volume
mM	milliMolar
MIC	Minimum Inhibitory Concentration
TSA	Tryptone Soy Agar
TSB	Tryptone Soy Broth
BA	Blood Agar
LSA	Listeria Selective Agar
MC	Mac Conkeys Agar
S(D)DW	Sterile (Double) Distilled Water
MSM	Minimal Salts Medium
MRS	deMann Rogosa Sharpe Broth
SIF	Simple Synthetic Intestinal Fluid
CIF	Complex Synthetic Intestinal Fluid
IF	Ileostomy Fluid
HPLC	High Performance Liquid Chromatography
GC	Gas Chromatography
ACN	Acetonitrile
THF	Tetrahydrofuran
LDH	Lactate Dehydrogenase
ADH	Alcohol Dehydrogenase
GSH	Glutathione
NAD	Nicotinamide dinucleotide
NADH	Reduced Nicotinamide dinucleotide

CHAPTER 1

INTRODUCTION

1.0 INTRODUCTION

1.1 Garlic

Natural products of plants have provided a valuable source of pharmacological agents with important bioactivity. The Genus *Allium* contains over six hundred species including; onions, chives, shallots, rakkyo, kurrat, leeks and garlic as well as various other ornamental and wild species. These have been grown for many centuries for their characteristic, pungent flavour and medicinal purposes.

Garlic (*Allium sativum L.)* has been widely used for medicinal purposes for thousands of years and traditionally invoked as a protective agent against stroke, atherosclerosis and coronary thrombosis (Fenwick & Hanley, 1985a, b, c; Kleijnen *et al.*, 1989; Lau, 1989). More recently, garlic has been shown to be effective as an anticancer agent (Criss *et al.*, 1982; Belman, 1983; Nishino *et al.*, 1989; World Cancer Research Fund, 1990; Dorant *et al.*, 1993) and a constituent of garlic, allitridium, shown to have spermicidal activity (Qian *et al.*, 1986). Garlic has also been implicated as an antimicrobial agent (Walton *et al.*, 1936), having a wider spectrum of activity than other antibiotics exhibiting: bacterial (Subrahamayan *et al.*, 1958; Tynecka & Gos, 1973, Elnima *et al.*, 1983), antifungal (Tansey & Appleton, 1975; Barone & Tansey, 1977; Adetumbi *et al.*, 1986), and antiviral properties (Tsai *et al.*, 1985; Weber *et al.*, 1992). As well as the health benefits, garlic has also been shown to be highly effective as an insecticide in the form of garlic oil (Amonkar & Reeves, 1970).

1.1.1 History Of Garlic

An Egyptian papyrus dating around 1500 BC described 32 garlic preparations which were active against a variety of complaints, including headache, body weakness and throat disorders. The Charaka-Samhita, an Indian medical manuscript written in the first century A.D, attributed the widespread curative properties of both garlic and onion claiming they had diuretic properties beneficial to the digestive tract. Pliny the Elder (79 A.D) devised 61 garlic-based remedies against conditions such

as rheumatism, haemorrhoids, ulcers and loss of appetite. Hippocrates in the 1st century recommended garlic as a treatment for pneumonia and suppurating wounds, however it causes flatulence, a feeling of warmth on the chest and a heavy sensation in the head, it excites anxiety and increases any pain which may be present. Nevertheless it has the good quality that it increases the secretion of urine.

The writings of Pliny and Hippocrates played a significant influence on medicine in the Middle Ages and thereafter. In 1626, Mattiolus recommended garlic against stomach chills and colics. In Dublin, at the turn of the last century, garlic in the form of inhalants and ointments was used successfully against tuberculosis. In Japan and Russia, garlic based antibiotics were used to combat an outbreak of influenza. Believers in natural medicine have reported the myriad benefits of garlic, described as the bulb with the "miracle healing powers" or the "wonder food" (Fenwick & Hanley, 1985a).

It is, however, only recently that such claims have been subjected to scientific scrutiny and studies into the physiological and therapeutic effects of garlic, its products and essential oil have only been conducted since the early part of this century. Moreover these have been limited by the lack of knowledge about the nature, origin and the interrelationships of the chemical components in the fresh tissue, processed product or essential oil.

Investigations made by chemists more than a century ago, established that cutting a garlic bulb releases a number of low molecular weight organic molecules that incorporate sulphur atoms in bonding forms rarely encountered in nature. Semmler (1892) established the importance of diallyl disulphide and diallyl trisulphide in the flavour of garlic distillate. Work performed by Cavallito and co-workers (1944a, b) and Stoll & Seebeck (1951) during antimicrobial investigations was crucial in the further development of an understanding of the chemical and biochemical

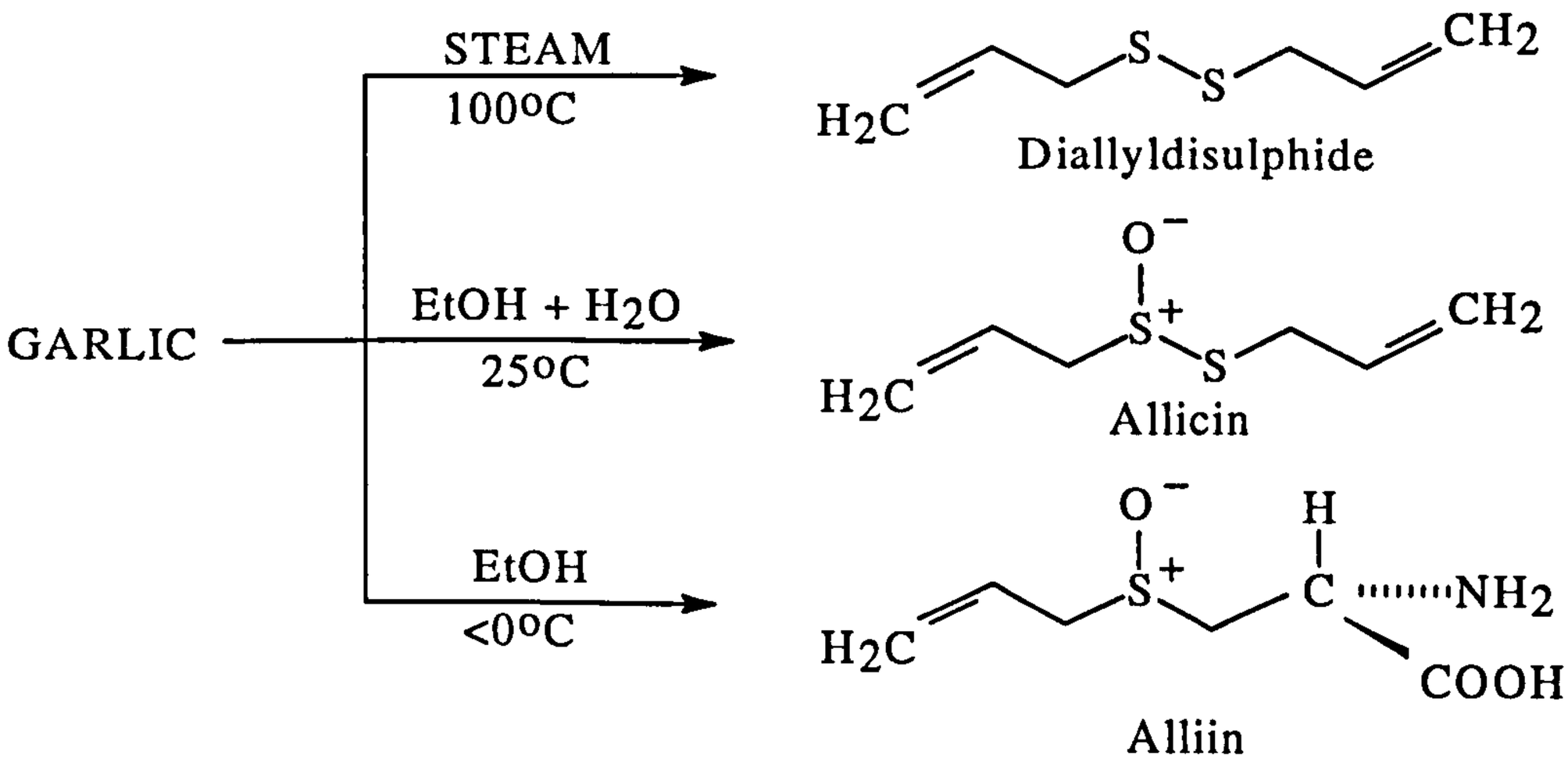
basis of the therapeutic effects.

Initial studies on the antibacterial properties of crushed garlic juice by Walton *et al.* (1936) indicated the biologically active components to be di(2-propenyl) sulphide and elemental sulphur derived from polysulphides. The actual nature of the antimicrobial activity of garlic was established later by Cavallito & Bailey (1944), they subjected an aqueous ethanolic extract of garlic cloves to steam distillation at 100°C under reduced pressure, isolating a water soluble substance which they named "allicin". Allicin is a colourless, unstable compound having the empirical formula $C_6H_{10}OS_2$ and molecular weight of 162. They reported that aqueous solutions of allicin had an antibacterial action at concentrations as low as 1:125,000 while di(2-propenyl) sulphide and polysulphides possessed no such activity.

1.1.2 Chemistry Of Garlic

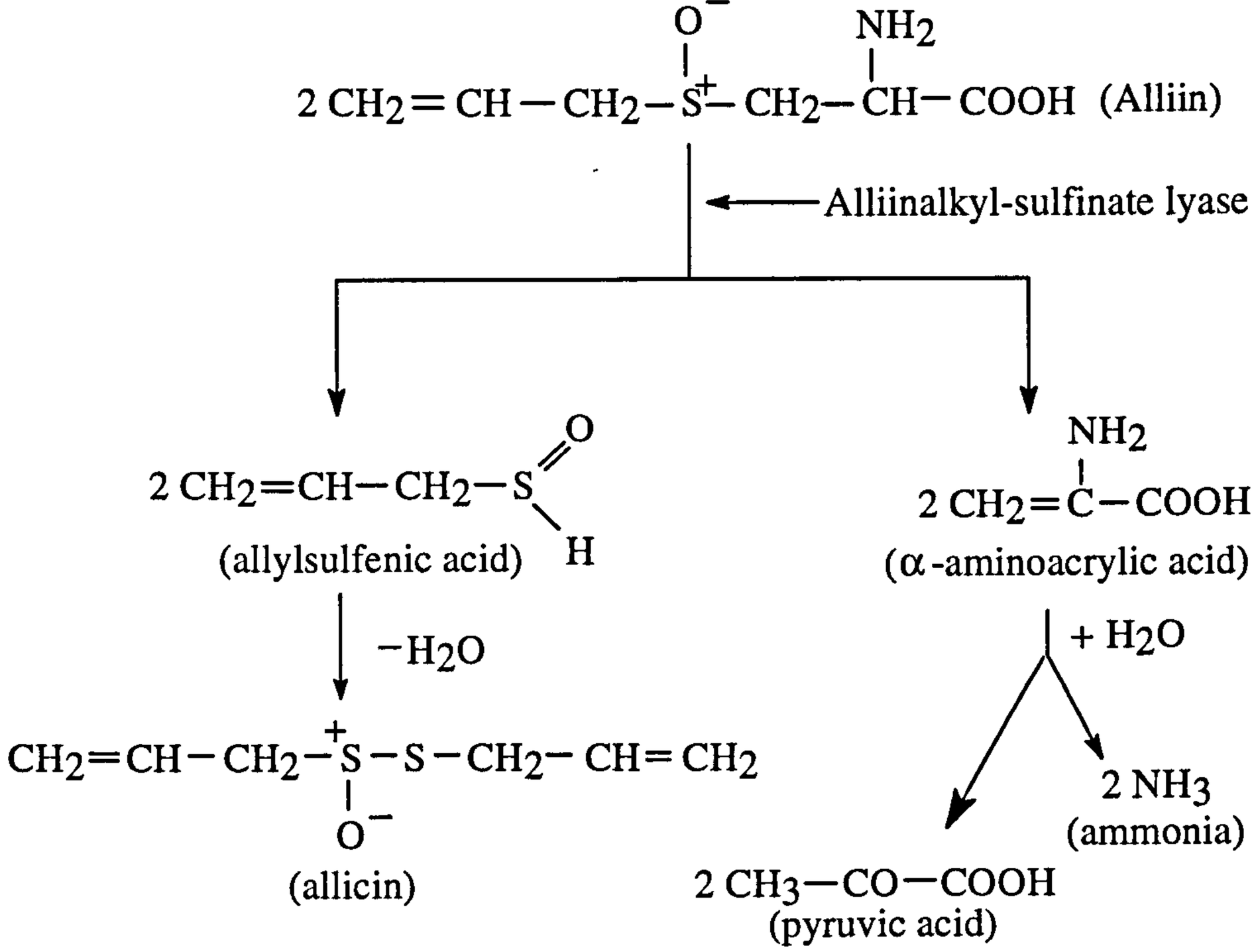
Stoll & Seebeck (1948), showed that garlic oil contains the oxide of diallyl disulphide known as allicin (allyl 2-propenethiosulphinate) and a mixture of aliphatic mono and poly sulphides (cysteine sulphoxides and thiols, of which diallyl disulphide is the main sulphur containing component) and the principal substance isolated by steam distillation as shown in Figure 1.1.2a.

Figure 1.1.2a Principal Components From Various Preparations Of Garlic



Allicin is not found in intact plants but is formed by the action of the enzyme alliin alkyl-sulphinate lyase (alliinase) on the non protein amino acid (+)-S-allyl-L-cysteine-S-oxide (Alliin) (Figure 1.1.2b). Alliin itself has no bactericidal activity (Stoll & Seebeck, 1948).

Figure 1.1.2b Enzymic Action Of Alliinalkyl-Sulfinate Lyase (Alliinase)



In intact plants the amino acid and enzyme are stored in separate cellular compartments within the cloves. Upon injury the barriers are broken and alliinase (enzyme) catalyses the β-elimination of alliin (amino acid) to yield pyruvate and ammonia (in equimolar quantities) as well as allylsulfenic acid, two molecules of which spontaneously react to form allicin.

Initial studies on garlic allinase by Stoll & Seebeck (1951) showed the crude cell-free preparation to be of unspecified purity with a broad spectrum pH optimum (5-8) and a temperature optimum of 37°C. Mazelis & Crews (1968) demonstrated

the stimulation of the enzyme by pyroxidal phosphate and that the addition of cysteine or hydroxylamine inhibited the action of the enzyme. They showed that alliinase catalyses the reaction of alliin to allylsulfenic acid and the subsequent conversions to α-aminoacrylic acid and allicin proceed non-enzymatically at a high rate.

Allicin is not a stable compound, readily transforming into secondary products of various sulphides that contribute to the characteristic flavour and odour (Brodnitz *et al*, 1971; Block, 1985; Yu & Wu, 1989). One such pathway requires the combination of three molecules of allicin to form two molecules of 4,5,9-trithiadodeca-1,6,11-triene 9 oxide known as ajoene (See Appendix 1). In addition the decomposition of allicin yields thioacrolein, a highly reactive compound which dimerizes to produce two cyclic compounds 2-vinyl-[4H]-1,3-dithiin and 3-vinyl-[4H]-1,2-dithiin (See Appendix 1).

The commercial production of garlic oil involves the use of heat to generate the volatile sulphides from garlic homogenates, therefore it is of interest to investigate the influence of heating on garlic homogenates with respect to the allicin and sulphide composition (Barlow & Wang, Personal Communication). It was found that allicin disappeared within 30 minutes of heating at 100°C and completely changed to various sulphides (allylmethyl disulphide, diallyl disulphide and allylmethyl trisulphide being the major three). This indicates that any heating process will convert all allicin to sulphides, so that any products formulated only with garlic oil are unlikely to contain any allicin. Hence, Cavallito's statement (1944) that steam distilled garlic oil (containing di(2-propenyl) sulphide and polysulphides) showed practically no antibacterial activity is true, if allicin is indeed the only antibacterial agent (See next section).

Yu & Wu (1989) demonstrated that pH adjustment from pH 5-8 (as stated by Stoll & Seebeck, 1948), during preparation of an aqueous garlic extract produced increasing amounts of diallyl disulphide, propylallyl disulphide and diallyl sulphide with increasing pH values. Formation of diallyl trisulphide, methylallyl trisulphide and *trans*-1-propenyl methyl disulphide were favoured by neutral conditions, whereas formation of allyl disulphide, methylallyl sulphide and methylpropyl disulphide were favoured around pH 9.0. It has been suggested that in expressed garlic juice the activity of di(2-propenyl) thiosulphinate is potentiated by the other garlic compounds present (Kabelik, 1970). Reduction by cysteine and dithiothreitol to di(2-propenyl) disulphide (Cavallito *et al.*, 1944a, b; Johnson & Vaughn, 1969) decreases the antimicrobial effect but this is restored following oxidation with hydrogen peroxide (Kabelik, 1970).

1.1.3 Antimicrobial Nature Of Garlic

1.1.3.1 Antibacterial Activity

The antimicrobial properties of garlic were first described by Pasteur (1858), confirming garlic's age-old reputation as a purifying remedy. Lehman (1930) found that extracts of the plant inhibited the growth of both *Escherichia coli* and *Salmonella typhimurium*. Johnson & Vaughn (1969) reported the effects of freshly reconstituted onion and garlic powders on *S. typhimurium*. Cultures of *E. coli* were found to be susceptible to garlic but more resistant to onion than were comparable cultures of *S. typhimurium* and a maximum bactericidal effect was observed at 5% (onion powder) and 10% (garlic powder). Comparison of the disulphides present indicated that di(2-propenyl) disulphide and dipropenyl disulphide were the most active, both were bacteriostatic at 0.1% level. Subrahmanyan *et al.* (1957a, b) showed garlic to be the most potent of 13 different spices, inhibiting the growth of *Shigella sonnei*, *Staphylococcus aureus* and *E. coli* at a 10mg level.

The antimicrobial effects of garlic oil on food poisoning bacteria were investigated by Mantis *et al.* (1978; 1979). They showed that a <1% garlic extract had no effect on the growth of *Staph. aureus*, intermediate levels >2% were inhibitory on small inocula and at high levels (5%) the garlic extract was bactericidal. *Clostridium perfringens* was inhibited by concentrations of 10% (small inocula) and 2% (large inocula) and a 1% garlic extract inhibited *Lactobacillus plantarum*, while 2-5% proved bactericidal. De Wit *et al.* (1979) showed that garlic oil partially inhibits toxin production by *Clostridium botulinum* type A in meat, but showed no effects on *C. botulinum* type B or E toxins. Since industrial meat products may contain several types of *C. botulinum*, it was suggested by these workers that caution be taken against the use of garlic for toxin inhibition. Beuchat (1976) demonstrated that garlic powder exhibited slight inhibitory effects against the food poisoning organism *Vibrio parahaemolyticus*. In 1974, a German patent was filed citing the effectiveness of garlic and onion mixtures in food as inhibitors of microbial growth and spoilage.

Small *et al.* (1947) showed that antibacterial activity of garlic was not confined to allicin alone but was a general property of aliphatic alkyl esters of alkyl thiosulphenic acids, all of which were shown to possess strong activity against both Gram +ve and Gram -ve bacteria. Comparative investigations of extracts from garlic and onion by Dankert *et al.* (1979) against Gram +ve and Gram -ve bacteria and yeasts, showed that garlic juice was inhibitory to all the organisms tested, while extracts obtained from the onion had no effect on Gram -ve bacteria.

The greater activity of the garlic extract was attributed to its higher ethereal oil content and the greater activity of di(2-propenyl) thiosulphinate as compared to the dimethyl and dipropyl analogues present in onion juice. Steam distilled preparations of garlic oil containing the natural diallyl disulphide and diallyl polysulphides showed reduced antibacterial action when compared with isolated allicin (Cavallito *et al.*, 1945). However the antibacterial activity (or lack of activity) of sulphides

depends upon direct evidence and not the suggestion that allicin is the only antibacterial agent. It has been shown, that other garlic components possess antimicrobial activity and a number of disulphides have been examined, the most active being di(2-propenyl) disulphide and dipropyl disulphide, both of which were shown to be bacteriostatic at a 0.1% level (Dankert *et al.*,1979).

No sign of the development of garlic-resistant bacterial strains has been found. Klimek *et al.* (1948) showed that *Staphylococcus aureus* 209P (known to develop resistance to penicillin and streptomycin) developed very little (if any) resistance to di(2-propenyl) thiosulphinate. Garlic may therefore be more indiscriminate than modern antibiotics but considerably weaker. In comparative plate culture tests, 0.1ml of fresh garlic juice was at least as effective as 6µg of penicillin, 100µg of streptomycin, 50µg of chloramphenicol or 30µg of tetracycline (Fulder, 1990). Comparable resistance tests do not appear to have been performed with garlic oil.

1.1.3.2 Antiviral Activity

Garlic has been shown to possess antiviral activity but the compounds responsible have as yet not been identified. The traditional antiviral uses of garlic include treatment of chicken pox, measles, the common cold and influenza (Fenwick & Hanley, 1985c). Tsai *et al.* (1985), showed that a commercial Chinese garlic extract was effective against herpes simplex virus type 1 and influenza B virus but not coxsackie B1 virus. Nagai (1973), showed that administration of the extract at the time of viral infection had no effect, while administration 15 days before experimental infection with influenza virus AO/RR8 provided protection. It has been suggested that antiviral properties of garlic may be due to; a direct antiviral effect, immune modulation, or a combination of both these events. Weber *et al.* (1991), showed that virucidal activities of commercial products were dependent upon their preparation process and those products producing high levels of allicin and other thiosulphinates provided the best virucidal activity.

1.1.3.3 Antifungal Activity

Extracts of garlic have also been shown to inhibit the growth of many plant pathogenic fungi and yeasts. Smalley & Hansen (1962) showed that growth of *Penicillium* species (that normally attack garlic) were less inhibited by crude extracts of garlic than were penicillia from other host species (*P. digitatum*, *P. expansum* and *P. granulatum* were all strongly inhibited by garlic). Further work indicated that alliinase and alliin, isolated from garlic had no effect individually on these strains, but in combination strongly inhibited all three *Penicillium* strains. Manning & Moore (1977) identified the active principle as allicin.

Tansey & Appleton (1975); Appleton & Tansey (1975) examined the effects of garlic on 139 species of fungi, approximately half were zoopathogenic, 58 species exhibited no growth when subjected to 0.005ml of extract in 5ml of malt broth, a further 15 species showed reduced growth when compared to controls. Agrawal (1978) showed that bulb extracts of garlic were found to be more active than the root extracts. Caporaso *et al.* (1983) found no detectable antifungal activity in the urine excreted following ingestion of 25ml garlic extract, hence concluding that orally administered garlic would be of limited use in the treatment of human fungal infections.

1.1.3.4 Insecticidal Activity

In India and Pakistan, garlic is used as an insect repellent for preserving food grains. The first controlled studies on the insecticidal activity of garlic were performed by Amonkar & Reeves (1970), who examined the effects of a crude methanol extract of dehydrated garlic and a steam distilled fraction against mosquito larvae. Several studies have been conducted into the biochemical basis of larvicidal activity. Amonkar & Banerji (1971) showed the insecticidal property of garlic oil to be attributed to two of its components; diallyl disulphide and diallyl trisulphide. George *et al.* (1973) showed that both garlic oil and diallyl disulphide inhibit

protein synthesis in mosquito larvae.

1.1.4 Antimicrobial Mechanisms

The effects of allicin are generally attributed to its interaction with biological sulphydryl containing systems (Cavallito *et al.*, 1945). Wills (1956), by enzyme inhibition studies, showed that alliin did not inhibit any of the enzymes tested, whereas allicin at 0.0005M inhibited nearly all sulphydryl enzymes tested. Thus indicating that the -SO-S grouping is essential for the bactericidal action of allicin, while the -S-S- (disulphide), -S- (sulphide) and -SO- (sulphoxide) groupings were not effective. Wills also showed that sulphydryl enzymes could be protected against inhibition of allicin by the addition of cysteine or glutathione. Evidence therefore suggests that allicin probably inhibits the enzymes present in the periplasmic space of microorganisms by interacting with the sulphydryl containing groups of proteins. If the latter are necessary components for the growth and proliferation of microorganisms, these processes will be inhibited by the presence of allicin (Cavallito *et al.*, 1944b; Tynecka & Gos, 1973).

The fungicidal activity of aqueous garlic extracts against plant pathogenic fungi was shown also to be due to allicin. Manning & Moore (1977) showed that the active principle was heat labile and active over a pH range of 2-10, in some cases allicin exhibited fungistatic rather than fungicidal activity. Again, the antimicrobial activity was thought to be due to allicin's reactivity toward essential -SH groups leading these workers to suggest interference with disulphydryl reductase functions of SH groups within the cell. Barone & Tansey (1977) suggested that the mechanism of anticandidal action of allicin involved the formation of mixed disulphides with protein sulphydryl groups.

Adetumbi *et al.* (1986) showed that aqueous garlic extracts inhibited protein and nucleic acid synthesis of *Candida albicans* to the same extent as growth, however as

with Ghannoum (1988), they showed that allicin causes complete inhibition of lipid biosynthesis leading to cell wall damage in *Candida albicans.* Feldberg *et al.* (1988) showed that although it is unlikely that allicin can function as a general sulphydryl reagent, the inhibition of macromolecular synthesis in allicin-treated cultures suggests more specific cellular functions are inhibited by allicin in growing cells. They indicated that allicin specifically inhibited RNA synthesis in bacteria.

1.1.5 Commercially Available Garlic Preparations

Commercial garlic preparations can be placed into three main categories; 1) oil of steam distilled garlic; 2) dried garlic powder AND 3) odourless garlic extract.

A) Garlic oil products - garlic is mashed and steam distilled. The process allows conversion of all alliin to allicin, which immediately breaks down to the various sulphides (as explained earlier). These are collected in a condensate as essential oil of garlic, which has similar constituents to crushed fried garlic. The oil is suspended in vegetable oil and encapsulated. This method was pioneered nearly 50 years ago by Dr Höfels. The capsules release odour when digested although this has been reduced by an enteric coating. One disadvantage of G.O products is the difficulty in standardisation and therefore many products are under dosage, it is known that 1g of garlic produces 1-2mg oil, that is 3-8mg oil per clove of 3-4g, whereas some garlic oil capsules contain 0.66mg oil, requiring 10 capsules (6.6mg oil) for a minimum one-clove recommended daily dose. However a high-dose garlic oil preparation (Cardiomax) is widely available with a satisfactory dose of 4mg fresh oil per capsule.

B) Dried garlic powder products - There are several types available, but those marketed in Europe are obtained by slicing garlic, freeze-drying the slices, milling and tabletting. This process releases only small quantities of allicin. The powder contains mostly alliin and alliinase precursors which are uncombined in the dry

state. It is assumed that the precursors combine on mixing and wetting in the alimentary tract to release allicin internally, and for this reason "odour-controlled" products contain very little odour unless chewed or dissolved. The rationale for these preparations is hypothetical at present, as allicin creation in the intestine is as yet untested. 1g of dried garlic in tablet form needs to be consumed to reach the minimum one-clove recommended daily dose, since fresh garlic has a water content of approximately 66% (Lawson *et al.* 1991c). The enteric-coated G.P tablet marketed as Kwai contains only 100mg of G.P euivalent to 300mg of fresh garlic.

C) Totally odourless garlic extract products - Garlic is chopped and aged in alcohol, after which it is extracted and dried. The resulting extract is totally odourless even on dissolving. The active ingredients of garlic are known to be odourous, and there is little evidence that this type of preparation is medicinally effective. Lawson *et al.* (1991c) has shown that no garlic sulphide compounds are present in this preparation.

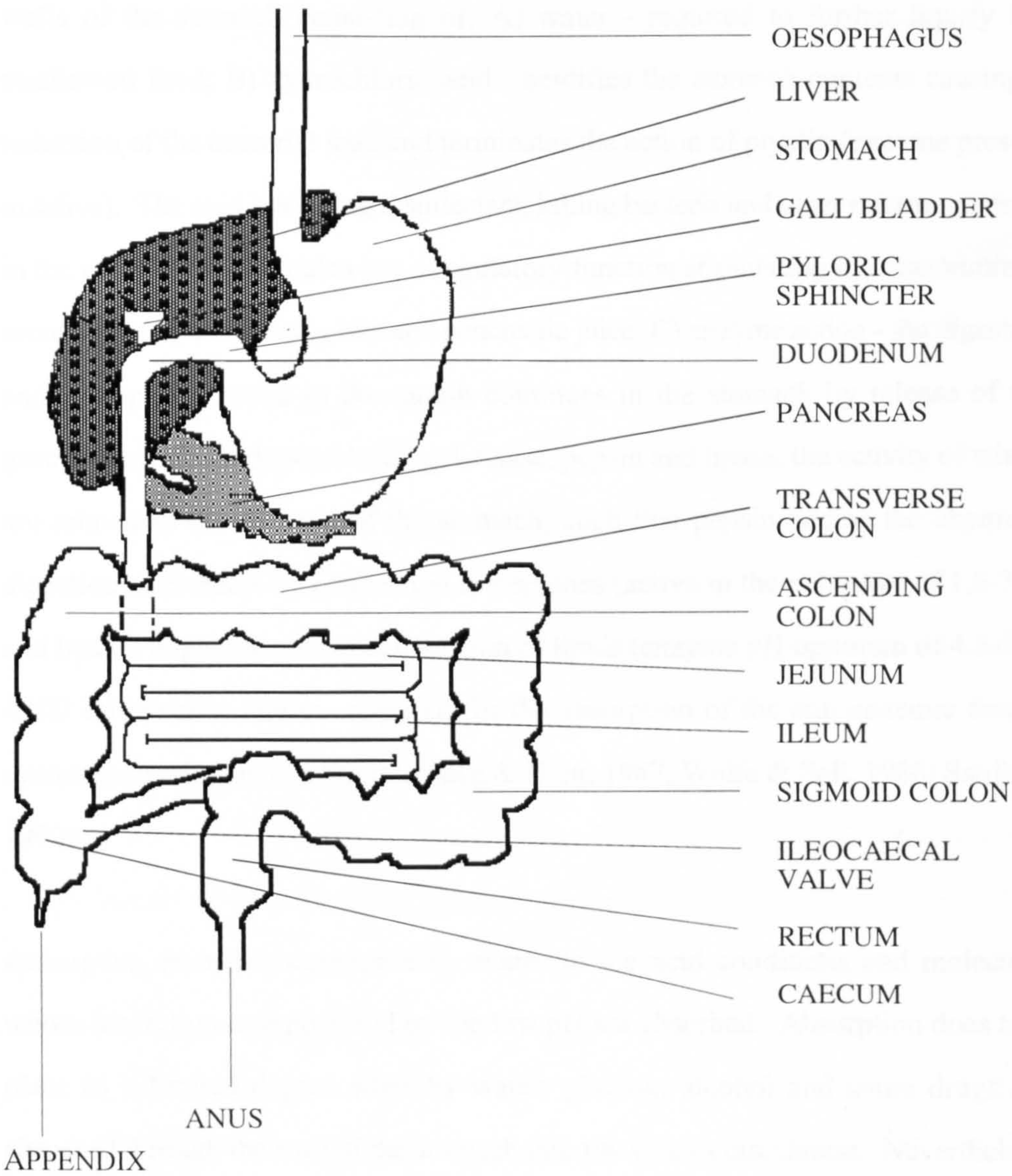
Over recent years, a variety of garlic based health products have become available on the market. The claimed health advantages of these products has generated a great interest in and a need for analytical methods to evaluate their quality. In order to evaluate these preparations standardisations are necessary. Various types of garlic powder have been standardised with respect to alliin or allicin and quantitative methods of determination of these products by HPLC and GC have been published (Saghir *et al.*, 1964; Jansen *et al.*, 1987; Mochizuki *et al.*, 1988; Iberl *et al.*, 1990a, b; Hughes & Lawson, 1991; Lawson *et al.*, 1991a, b, c; Lawson & Hughes, 1992).

In order to understand the investigations into the enteric antimicrobial activities of the garlic products undertaken in this thesis, it is important to have a full comprehension of the environment and microbiology of the gastrointestinal tract.

1.2 The Gastrointestinal Tract

The GI tract consists of a long, continuous, muscular tube beginning at the mouth, running through the body and ending at the anus (Figure 1.2a). The structure and function of the intestinal tract will firstly be discussed, followed by its associations with intestinal flora.

Figure 1.2a Structure Of The Gastrointestinal Tract



1.2.1 The Various Environments Of The Gastrointestinal Tract

1.2.1.1 Physiology Of The Stomach

Food leaving the mouth, homogenized and mixed with saliva passes down the oesophagus into the stomach. The stomach acts as a temporary reservoir for food, it can accomodate large (5 litres) or small (0.5 litres) volumes of material, allowing digestive juices time to act on the different food substances. Acid and enzymes are secreted into its lumen. About 2 litres of gastric juice are produced daily, with a flow rate of 40ml/hr (basal) and 100ml/hr (active), secreted by special glands in the walls of the stomach consisting of; A) water - required to further liquify the swallowed food; B) hydrochloric acid - acidifies the stomach contents causing a reduction of the bacterial load and terminates the action of ptyalin (enzyme present in saliva). The acid is a strong disinfectant, killing bacteria and other microorganisms in the ingested food. It also has a regulatory function stimulating the duodenum to secrete hormones releasing bile and pancreatic juice; C) enzyme action - the digestion and absorption started in the mouth continues in the stomach by release of the gastric enzymes; endopeptidases, gelatinase, pepsin and lipase, the activity of which are related to the low pH of the stomach, such that pepsin begins the chemical digestion of proteins by conversion to peptones (active in the pH range of 1.8-3.5) and lipases begin the chemical digestion of lipids (enzyme pH optimum of 4.5-5.5) AND D) intrinsic factor - required for the absorption of the anti-anaemic factor, cyanocobalamin, vitamin B_{12} (Howden & Hunt, 1987; Wolfe & Soll, 1988; Sanford, 1992).

Absorption from the stomach also relates to the acid conditions and molecules whose ionization is suppressed by the low pH are absorbed. Absorption does take place to a limited degree whereby water, glucose, alcohol and some drugs are absorbed through the wall of the stomach into the venous circulation. Nevertheless, in the stomach considerably more secretion than absorption occurs and food entering the small intestine has an increased water content and is osmotically better balanced.

The ingested material is modified in the stomach to produce a fairly uniform product as regards pH, osmolality, consistency and temperature, known as chyme. Chyme is passed into the duodenum in small quantities when the pyloric sphincter relaxes and the muscular walls of the stomach contract.

1.2.1.2 Physiology Of The Small Intestine

Most digestion and absorption occurs in the small intestine and its structure and activities tend to maximize efficiency in these respects. In the omnivorous human, the small intestine is of medium length (c. 6m), and can be divided into the upper, mid and lower areas, known as the duodenum (short <1m), jejunum (c. 2m long) and ileum (c. 4m long), respectively. The ileum itself is divided again into the distal and terminal areas. Active flow rates of material within each of these areas are; 560ml/hr in the duodenum to mid jejunum, 57ml/hr in the late jejunum and 36ml/hr in the ileum.

Movements of the small intestine mix chyme with the digestive secretions, bringing fresh chyme into contact with the absorptive surface of the microvilli, and propel chyme toward the colon. The volume of material entering the intestine is very large, ingested material (food and drink), contribute only a minor component. It has been estimated for man, that of the 7 litres of liquid entering the duodenum each day, only 2 litres are contributed by the diet (Sanford, 1992). Other contributors include the secretions of the gastric mucosa, intestinal mucosa, the pancreas and the gall bladder. This swamping of dietary substances ensures a constant environment and thus enables the digestive enzymes to operate under controlled conditions.

Carbohydrates, proteins and fats are all digested (by enzymatic action) and absorbed (some by active transport) from the small intestine. It is known that the duodenum and upper jejunum have the highest capacity to absorb sugars, proteins (in the form of small peptides) and lipids with the capacities of the lower jejunum and ileum

being progressively less. Pancreatic juice is a major source of enzymes and includes the buffering capacity required to neutralize gastric acid and optimize the conditions of enzyme action. Enzymes present in the pancreatic juice include carboxypeptidases, an amylase, various lipases, ribonuclease, deoxyribonuclease, collagenase, elastase as well as trypsinogen and chymotrypsinogen, the latter two are inactive enzymes prior to contact with enterokinase, from intestinal juice, converting them to active trypsin and chymotrypsin. Enzymes from *succus entericus* (intestinal juice) include enterokinase, sucrase, maltase and lactase. Products of the action of these enzymes are then available for further digestion and absorption. Amylase present converts all polysaccharides (starches) not affected by ptyalin to disaccharides (sugars), which occur at the mucosal surface and these complete the hydrolysis of carbohydrates.

The digestion of fats involves not only lipases, which convert fats to fatty acids and glycerol, but also bile acids, cholic acid and chenodeoxycholic acid (water soluble derivatives of cholesterol), secreted by the liver these solubilize fats and products of fat digestion and facilitate their absorption, conjugated either with glycine or taurine to form chenylglycine, chenyltaurine, cholyglycine or cholytaurine. These are converted into the secondary bile acids; lithocholic acid and deoxycholic acid a process performed by bacterial metabolism in the intestine. Specific mechanisms exist to potentiate the absorption of some amino acids and some monosaccharides, such as glucose. Bile acids are recirculated and vitamin B_{12} is absorbed by specific transport systems. Particular mechanisms exist for various essential substances.

Partition between mucosa and intestinal contents and passive diffusion make an important contribution to absorbtion. The structure of the small intestine maximizes the area available for the absorption to occur. The surface of the mucosa is convoluted and folded, from these folds and convolutions spring the finger-like villi, the surface of which are covered with the absorptive cells. The surface of

these cells which face into the intestinal lumen, are themselves covered with microscopic protuberances, the microvilli.

1.2.1.3 Physiology Of The Large Intestine

Residues and debris left after digestion and absorption in the small intestine pass through the ileocaecal valve into the large intestine awaiting elimination from the body. The large intestine, includes the caecum, appendix and colon. The alimentary tract terminates with the rectum and anal canal. It is approximately 1.5m in length. The colon can be divided into four regions, the ascending (15cm), transverse (50cm), descending (25cm) and sigmoid (40cm) regions. Continuous with the sigmoid colon is the rectum (approximately 12cm long), which passes towards the anal canal.

The colon has a flow rate of 1-10ml/hr, receiving about 1.5 litres of chyme/day from the terminal part of the ileum. Most of the salts and water that enter the colon are absorbed; the faeces normally contain only about 50-100ml of water/ day. Muscular movements (segmentation) of the colon, assist this process by ensuring contact of the faecal matter with the absorptive surface. Bile salts and electrolytes from the food wastes are absorbed and the secretion of mucus to lubricate the passage of digesta also occurs in the large intestine (Davenport, 1985). The rectum acts as a reservoir for the faeces until it is convenient to evacuate from the anus.

1.2.2 Microbiology Of The Gastrointestinal Tract

The intestine of man harbours a complex mixed culture of microorganisms that develops as a result of the influence of intestinal physiology on the interactions between those bacteria from the environment that contaminate the body.

The processes involved in microbial establishment are complex, involving numerous successions of microbes and microbial/host interactions. Eventually dense, stable populations come to inhabit characteristic regions of the gut. Many factors, including diet, the rate of passage of digesta and the degree of acidity of the gut contents have been suggested as influencing the development of the flora (Giannella *et al.*, 1972; Luckey, 1972; Drasar & Hill, 1974; Finegold *et al.*, 1983; Cummings & Macfarlane, 1991). In most animals, microbial populations develop where stasis or slowing of the passage of digesta occurs as in the hindgut or foregut, an additional requirement is a pH of digesta close to neutrality, which excludes the stomach as a suitable environment.

1.2.2.1 Initial Colonisation Of The Gastrointestinal Tract

Development of microbial populations in the alimentary tract of humans commences soon after birth, with the initial inoculum usually derived from the vagina and external genitalia of the mother (Rotimi *et al.*, 1981; Stark & Lee, 1982). The initial period of bacterial colonization and adjustment of the colon, takes place over a period of about two weeks, during this period *E. coli* and *Streptococcus* appear in large numbers, typically 10^8-10^9 organisms/g faeces. *Bifidobacterium* then reach levels of 10^8-10^{11}/g by 4-7 days of age, *Clostridium* and *Bacteroides* may also appear at high levels initially, then may disappear (Willis *et al.*, 1973; Stark & Lee, 1982; Copperstock & Zedd, 1983).

Differences between breast-fed and bottle-fed infants have also been noted (Bullen *et al.*, 1976, 1977). The addition of food supplements to the diet of infants probably causes major shifts in the faecal flora, although this concept has never been studied in a controlled fashion (Stark & Lee, 1982) and the differences between breast-fed and bottle-fed infants are lost (Haenal, 1970). By about the second year of life major bacterial populations in faecal flora come to resemble those of adults (10^6-10^8 bacteria/g of faeces) (Stark & Lee, 1982; Copperstock &

Zedd, 1983). While the adult flora is characteristic to man, that of the neonatal mammal is common to a wide range of species since the milk diet produces a common environment in the gut. Characteristic of the gut flora of neonates are the low numbers of potentially pathogenic species such as *E. coli*. These low numbers are maintained by the inhibitory effects of specific antibody (mainly IgA) and several non-specific factors including the iron binding protein, lactoferrin.

1.2.2.2 Microbial Composition Of The Stomach

It is generally believed that the environment of the normal human stomach is relatively sterile; with only low counts of a variety of microorganisms. Studies have shown low counts (c.10^3 colonies/ml gastric contents) of α-haemolytic *Streptococci*, anaerobic cocci, *Lactobacilli*, *Staph. epidermidis* and *Candida albicans* (Giannella *et al.*, 1972). These low counts may represent oral and ingested organisms since counts tend to decrease to zero gradually several hours after meals (Smith, 1966; Drasar *et al.*, 1969). Giannella *et al.* (1972) showed that the gastric pH appears to play a major role in controlling the growth of organisms in the stomach and larger numbers of bacteria (c.10^6/ml) have been recorded in subjects with reduced gastric acidity.

Recent research has suggested a connection between gastric/duodenal ulcer and *Helicobacter pylori* associated with the stomach lining where it is generally believed that it can grow and proliferate. At the luminal face of the secreting cells in the stomach, the pH is low (0.8-1.0), hence bacteria such as *Helicobacter* must be able to tolerate the low pH conditions. It has been suggested that they survive by using their high content of urease to catalyse ammonia production from urea, thus enabling them to survive within an ammonia cloud forming a microniche.

1.2.2.3 Microbial Composition Of The Small Intestine

Microorganisms are frequently isolated from the contents of all regions of the small intestine and the population levels encountered in the small intestine of a normal individual are highly dependent on the location of sampling. The upper small intestine (duodenum) is generally sterile having low counts (0-$10^{4.5}$ colonies/ ml) of both aerobic (*Streptococci, Staphylococci, Lactobacilli* and yeasts) and anaerobic (*Streptococci* and *Lactobacilli*) organisms. These are known as fluctuating transients due to the flow rate. It should be noted that there is an almost complete absence of coliforms and *Bacteroides* (Gorbach *et al.*, 1967a, b; Drasar *et al.*, 1969; Skinner & Carr, 1974).

The lower small intestine (distal ileum) contains a flora that much more closely approximates that of the large bowel ($10^{3.5}$-$10^{6.3}$ colonies/ml) (Plaut *et al.*, 1967; Thadepalli *et al.*, 1974; Finegold *et al.*, 1983) with higher counts of coliforms and *Bacteroides* (Drasar & Hill, 1974; Duerdon, 1980; Finegold *et al.*, 1983). The terminal ileum appears to be a "transitional" zone between the relatively sterile (sparse flora) upper small intestine and the colon with its rich bacterial population and this may be related to the flow rate of fluid through the small intestine. The contents of the small intestine normally flow rapidly, possibly becoming static for an appreciable period only in the distal ileum. Thus, if any indigenous microbes colonize luminal habitats in the small intestine, they probably do so only in the distal ileum. Alternatively it may be related to the pH of the particular area of the small intestine (as most bacteria prefer slightly alkaline conditions), the jejunum has a pH of 6-7, while the pH of the ileum is 7.5.

The possibility exists that microbes isolated from the small intestine contents could be just merely passing down the bowel with digesting food, either from habitats above the small intestine or from outside the body. In the lower part of the ileum, isolated microbes could be derived from the rich biota of the caecum finding their

way via the ileocaecal valve into the small intestine. In either case, such organisms may be only transients in the lumen of the small intestine and not indigenous inhabitants.

1.2.2.4 Microbial Composition Of The Large Intestine

This part of the intestine contains the richest and most complex part of the intestinal flora (Cummings & Macfarlane, 1991). It should be noted that the supply of fermentable carbohydrate is an important factor limiting the growth of bacteria in the colon, as in other environments, bacteria that can transform available substrates most rapidly under the environmental conditions will proliferate in the greatest numbers. Bacterial products and other materials are absorbed from the large intestine but their importance is largely unclear (Macfarlane *et al.*, 1992).

In adult humans, the large intestine, including the caecum and colon, harbours complex microbiotas composed undoubtedly of both indigenous and allochthonous microorganisms. Enormous microbial populations can develop in the lumen of the large intestine, and especially that of the caecum, because these are areas of relative stagnation in the flowing stream that is the gastrointestinal tract. In these areas, the passage rate of luminal content does not exceed the doubling times of bacteria.

A substantial part of the indigenous microflora of the large intestine is associated with the gut wall in 2 ways; 1) a primary layer of bacteria adheres to epithelial cells, whereas several subsequent layers adhere to the primary one and/or to each other AND 2) bacterial layers are enveloped in what is usually described as mucus, even though the nature of this material and its origin (either from host or bacteria) have not been determined.

Across the ileocaecal valve, the change in the numbers and types of microorganisms that can be seen is dramatic. The main change is an increase in anaerobic populations, *Bacteroides*, anaerobic *Lactobacilli*, and *Clostridia* become the major constituents of the microflora, outnumbering the aerobic and facultative flora such as coliforms by 1,000:10,000:1. It is known that these microorganisms co-exist without one or a few becoming dominant and displacing the others (Freter *et al*, 1983; Cummings & Macfarlane, 1991). A range of different nutritional types of bacteria exists in the large bowel, reflecting the great variety of substrates available for growth (Freter, 1983). These include saccharolytic organisms which participate in breakdown and fermentation of complex carbohydrates (Vercellotti *et al*, 1977), bacteria that degrade proteins, peptides and amino acids (Macfarlane *et al*, 1986), methanogens and other bacteria that grow on the intermediate products of fermentation, such as hydrogen, lactate, succinate and ethanol (Gibson *et al*, 1988 a, b).

It has been shown that microbial counts in the transverse colon are on average 2-3 log values lower than in stool samples and bacterial counts in the terminal ileum are even lower with relatively few anaerobes (mean count $10^{4.4}$ colonies/g) (Gorbach, 1971). Bacterial counts in the faeces can average at least 10^{11} colonies/g (Moore & Holdeman, 1974). The highest counts obtained from stool specimens, showed the mean anaerobic count to be $10^{9.6}$ colonies/g and the mean coliform count to be $10^{7.5}$ colonies/g (Finegold *et al*, 1983), indicating that the facultative anaerobes (coliforms) although being thought of as characteristic organisms of the intestine, typically occur in numbers that are 2-3 orders of magnitude lower than those of the strict anaerobes.

It has been shown by numerous workers that microbes are associated with the intestinal epithelium in three different types of habitat; luminal, epithelial and the crypts of Lieberkühn. The natural intestinal microorganisms may associate with

epithelial surfaces in the habitats they occupy as may the pathogenic microorganisms. In the latter, the association is usually only temporary caused by a serious change in the regulation of the ecosystem, and can be a step in the pathogenesis of disease caused by the microbe (Leach *et al.*, 1973; Luckey, 1974; Freter, 1975). Dubos *et al.* (1965) and Savage *et al.* (1968) showed that the non-secretory epithelium of the stomach and oesophagus is colonized by a microbial community. Savage (1972b) noted an intimate association of some microorganisms with the intestinal epithelial surfaces, some species showing a strong attachment. Much of this work on microbe-epithelial associations originates from that of Porter & Rettger (1940), who noted that *Lactobacilli* may associate closely with the epithelium of the stomachs of normal rats. Plaut *et al.* (1967) and Nelson & Mata (1970), have shown that there are associations with epithelial surfaces in the small and large intestines of humans.

The gastrointestinal tract of animals, even in perfect health, harbours a complex collection of microbes and the predominant types of these microorganisms and their population levels remain relatively constant as long as the environment and dietary conditions of the healthy host remain constant (Savage, 1977). The gastrointestinal ecosystem, however is not static, it is a dynamic open system. Nutrients from the host's diet enter at one end of the tract and waste products and microbes exit at the other end (Macfarlane *et al.*, 1992). Some of the substances released or generated in the ecosystem are absorbed by the gastrointestinal epithelium which is renewed constantly and secretions enter the ecosystem. Microbes multiply, metabolize, and die. Some microbes interact with other microbial types. However, although the gastrointestinal ecosystem is a dynamic system, it is in a steady state.

1.2.3 Factors Affecting The Microbial Composition Of The GI Tract

The types and population levels of microbes in the gastrointestinal tract, and the successions of these communities, are regulated by multifunctional processes. Some of the regulatory forces in these processes are exerted by the mammalian host, others by the microbes themselves (Drasar, 1974; Drasar & Hill, 1974, Freter, 1975b). The factors that regulate this steady state can be divided into 2 categories; a) ALLOGENIC FACTORS - which originate outside of the ecosystem (such as the hosts diet) AND b) AUTOGENIC FACTORS - which arise within the ecosystem (such as microbial products that influence growth or other microbes).

1.2.3.1 Forces Exerted By The Host, Its Diet And Environment

1.2.3.1.1 Diet

The nature of a meal affects the gastric emptying which indirectly affects the distribution of bacteria within the small intestine. Although diet is important in determining the qualitative and quantitative composition of the intestinal microflora it is difficult to ascertain experimentally. A problem is that the composition of the normal intestinal flora may be influenced by many factors such as age, diet and geographical residence (Drasar & Barrow, 1985). Studies of people's diet in various countries performed by Drasar & Hill (1974) demonstrated differences in relative numbers of various groups of bacteria which may reflect the effect of the diet. It was shown that people living on a mixed "Western" type of diet (for example Britain and US) had greater *Bacteroides* and fewer enterococci and other aerobic bacteria than people living on a native, largely vegetarian diet in Africa and Asia.

1.2.3.1.2 Environment

A temperature optimum for growth of about 37°C is undoubtedly an asset to microbes colonizing any habitat in the intestine. Likewise, the ability to grow anaerobically is an advantage to microbes colonizing habitats in any location in the

tract (Onderdonk *et al.*, 1976).

Hydrogen ion concentration in terms of the gastric acid, is a major factor dictating what types of microbes can colonize habitats in the stomach (Gray & Shiner, 1967; Maffei & Nobrega, 1975; Howden & Hunt, 1987).

Peristalsis (by dictatory flow rate) is a strong influence preventing microbial communities from developing in the lumen of the upper and middle regions of the intestine (Savage, 1972b; Drasar & Hill, 1974). Clearance of bacteria by peristalsis was demonstrated by Dixon (1960), who showed that alterations in intestinal motility such as intestinal obstruction, stasis or fistulae lead to overgrowth of microorganisms within the lumen.

Repeated attempts have been made to demonstrate that bile acids are important forces regulating the microbiota in the small intestine (Gorbach & Tabaqchali, 1969). Some microbial types commonly isolated from human faeces are growth inhibited *in vitro* by certain unconjugated bile acids at low concentrations (Floch *et al.*, 1972; Binder *et al.*, 1975). Nevertheless, *in vivo*, the impact of bile acids on the composition of the microbiota in the small intestine is less well defined. The human intestine (as a consequence of changes in bile concentration in the intestine due to periodic secretion) rarely contains appreciable quantities of unconjugated bile acids (Mallory *et al.*, 1973). The presence of conjugated bile acids and their effect on microbes have been investigated (Drasar & Hill, 1974), but convincing evidence as to the effect on population levels has not as yet been proven.

Little evidence supports the hypothesis that an animal's immunological responses affect composition of the indigenous microbiota. Some bacterial inhabitants of the gastrointestinal tract can induce antibodies detectable in the serum and intestinal secretions (Brown *et al.*, 1972) or as produced by spleen cells of their hosts.

1.2.3.1.3 Antibiotic Therapy

Due to the complex intestinal flora, adults are normally extremely difficult to infect with enteric pathogens. Disturbances or removal of the flora (by antibiotics) increases susceptibility to colonization by pathogenic organisms. Thus the indigenous intestinal flora provides natural protection against infection by pathogenic bacteria and the protective mechanism is impaired when antimicrobial agents are administered. Antibiotics frequently produce profound changes in the composition of the human intestinal microflora, permitting overgrowth of resistant endogenous bacteria or colonization of exogenous organisms acquired from the environment.

Approximately 10% of antibiotic treatments are responsible for adverse gastrointestinal effects, among which diarrhoea is the most frequent. Once resistance is reduced by antibiotic administration, even relatively lower numbers of pathogenic organisms can produce serious infections in the host. Clearly, the integrity of the intestinal flora is important to the well-being of the host, and antibiotics, which upset it, should be used with extreme caution. An additional consequence of oral antibiotic administration is that commensal and pathogenic bacteria may become resistant to these drugs by mutation or by transferable drug resistance.

1.2.3.1.4 Surgery

It is known that surgical alteration of the lower gastrointestinal tract in terms of removal of the colon and construction of an ileostomy leads to marked changes in bacterial populations (Drasar & Hill, 1974). The flora of ileostomy effluents represents a unique ecological niche, corresponding neither to the normal ileal contents nor the intra-abdominal colon (Gorbach *et al.*, 1967c). Bacterial counts on ileostomy fluid by Gorbach *et al.* (1967c), Scarpino *et al.* (1969), Finegold *et al.* (1970) and Levine *et al.* (1970) indicated that cultivable anaerobes are greatly reduced with total counts generally 5-10 logs less than bacteria present in faeces, but were much higher than those obtained from aspirates of the normal ileum.

Bacteroides fragilis, the most common species in normal faeces, may even be completely absent in ileostomized patients (Finegold *et al.*, 1970). Levine *et al.* (1970) suggested that the recovery of anaerobes is related to the amount of colonic tissue present, and showed that if a portion of a large bowel is preserved by ileoproctostomy, the recovery of *Bacteroides* increases.

In summary, any dietary or environmental condition that adversely affects the optimal functioning of the host can thus affect the gastrointestinal ecosystem in a direct (such as change in nutrients entering the ecosystem) or indirect manner (such as some change in host physiology). The regulatory factors controlling the microbial populations in the ecosytem may be temporarily disrupted, and undesirable effects may be exerted on the host animal as a result (Tannock & Savage, 1974; Tannock, 1983).

1.2.3.2 Forces Resulting From Activities Of The Microorganisms

Alterations in the physiological function of the intestinal tract are among the most important effects produced by bacteria. All aspects of the intestine-digestive function, absorptive and secretory capacity and mucosal structure are influenced by bacterial action, but not all these changes result in an overt manifestation in terms of intestinal function.

Faecal type bacteria in the small intestine can alter host physiology in many ways; essential nutrients such as vitamin B_{12} may be directly bound by intestinal microbes and become unavailable for absorption by the intestinal mucosa (Tabaqchali *et al.*, 1966; Gorbach & Tabaqchali, 1969). Species of aerobic and anaerobic bacteria are capable of absorbing vitamin B_{12}. Intrinsic factor binding of vitamin B_{12} appears to inhibit bacterial uptake, however if enough binding of the intrinsic factor-B_{12} complex occurs in stagnant loops it will produce significant malabsorption.

Microbial populations in established climax communities undoubtedly exert strong direct forces to maintain the stability of the structure of their communities and the normal enteric microbes of man are specially adapted to the situation of the intestinal tract, most are anaerobes of the *Bacteroides* group although *E. coli*, enterococci, *Lactobacilli*, are common indicating that the normal flora are in a balanced state which tends to resist colonization from other bacteria.

The practical effect of these forces would be to exclude allochthonous microbes from niches in the habitat occupied by the established community. Such forces include death of other bacteria by bacteriocins (Savage, 1972a, b; Apella *et al*, 1992; Vandenbergh, 1993) antibiotics (Ducluzeau *et al*, 1976), nutritional competition (Savage, 1972a, b; Freter, 1975a), toxic metabolic end products such as volatile fatty acids (Freter & Abrams, 1972), H_2S (Freter, 1975a), competition for attachment sites and maintenance of low oxidation-reduction potentials.

Some microorganisms produce bacterial inhibitors, such that, there is a difference between *Lactobacilli* in the large bowel of breast-fed infants, which produce acid and other factors that inhibit the growth of other microbes. Together with antimicrobial components of human milk, these factors enable breast-fed infants to resist colonisation with other bacteria, such as pathogenic strains of *E. coli*. Bottle-fed infants, however lack protective *Lactobacilli* and are susceptible to those pathogenic strains of *E. coli* which may cause serious gastroenteritis.

Direct evidence as to whether nutritional competition operates in this ecosystem is also limited. Carbohydrates may be directly utilised by intestinal microorganisms colonising the small intestine. Gracey *et al* (1969) observed impaired absorption of glucose, galactose and fructose in infants with small intestinal stasis and bacterial overgrowth. Fat malabsorption has also been associated with bacterial overgrowth syndrome. Only small amounts of fat are directly metabolised by intestinal bacteria

and the major causes of this situation are disturbances in bile salt metabolism. Certain types of intestinal bacteria are capable of deconjugating bile salts, such as enterococci, *Bacteroides*, *Clostridia*, *Bifidobacteria* and *Veillonella* (Gorbach & Tabaqchali, 1969). As a consequence of bacterial overgrowth, the concentration of conjugated bile salts in the upper jejunum is reduced below the critical level necessary for micelle formation and this leads to malabsorption of fatty acids and monoglycerides (Tabaqchali *et al.*, 1966; Coleman, 1987). It should also be stated that intestinal bacteria may also alter the structure of bile acids by dehydroxylation, oxidation and reduction (Aries *et al.*, 1969).

Water and electrolyte transport in the small intestine may also be disturbed by bacterial colonisation. Bacteria such as *Vibrio cholerae* (Burrows, 1968) and *E. coli* (Gorbach & Etkin, 1970) produce exoenterotoxins which cause net fluid loss without penetrating or damaging the mucosa (Banwell *et al.*, 1971). Infection by *Salmonella* also causes fluid loss in the ileum but in association with direct mucosal invasion by the microorganisms. It has also been shown with *V. cholerae*, when they temporarily colonize an available habitat, that they induce antibodies that circulate in the serum, and secretory antibodies that enter the intestinal tract (Freter, 1975).

Since the oxygen potential varies according to the microbial population level, the microflora itself controls certain aspects of its own. Some groups of facultatively anaerobic bacteria, such as *Lactobacilli*, *Streptococci* and coliform bacteria are ubiquitous and are distributed throughout most of the tract. Obligate anaerobic bacteria such as *Bacteroides* and *Bifidobacterium* are confined to parts of the gut where Eh valùes are very low, such sites include the colon and caecum. The homeostatic mechanisms at work in the gut are able to minimize minor changes, for example although bacteria are able to destroy many of the pancreatic enzymes, such destruction seldom results in digestive insufficiency resulting from the large

excess of enzymes usually produced.

It has been known since the 1960's that some enteropathogenic bacteria such as *Vibrio sp* and certain serotypes of *E. coli* are able to multiply rapidly in the upper small intestine. They counteract the fast rate of flow of chyme present in this part of the tract by actively adhering to the intestinal epithelium though not in identical ways (Savage, 1970).

1.2.4 Microbial Analysis Of The Gastrointestinal Tract

1.2.4.1 Problems Of Models

Most bacteria (including species not indigenous to intestinal flora) are able to colonize germ free animals, even when these hosts are immunized (Shedlofsky & Freter, 1974), whereas the colonization of conventional animals or man is difficult to achieve. It has been generally assumed, therefore, that the major mechanisms that control the composition of the indigenous microflora of the intestine are a consequence of interactions among the numerous microbial species present.

One obvious difficulty in a study of such interactions involves the narrow limits of experimental manipulations possible within the intact animal. Consequently most investigations have resorted to working with *in vitro* models of bacterial interactions. *In vitro* model systems, cannot, however be relied on to reflect mechanisms of *in vivo* interactions, such that the interactions among a given group of microorganisms depends to a large extent on the environment in which they take place (Hentges & Freter, 1962). It should be noted that even a seemingly ideal *in vivo* model, the gnobiotic animal, cannot be accepted without question to duplicate mechanisms of bacterial interaction as they occur in the conventional animal harbouring a complete indigenous flora. It is well known that the intestinal contents of germ-free animals are entirely different from those of conventional animals. Consequently, interactions among bacteria in gnobiotic animals occur in an

environment that is unnatural and cannot *a priori* be accepted as being indicative of "normal" interaction.

1.2.4.2 Problems Of Sampling

Studies on the human intestine are complicated by the problems of obtaining samples of intestinal contents as most of the intestinal tract is normally inaccessible for examination. Much work on intestinal flora has been performed on faecal material (Duerden, 1980), however it should be recognised that the flora in faeces only represents the flora of the rectum.

Samples of small intestine/colonic contents are difficult to obtain and are affected by the procedures used in collection (as stated previously). Animals may be killed and the intestinal contents examined after death, but this method is not suitable for humans for obvious reasons. Several workers have obtained specimens from patients undergoing abdominal surgery by various methods, such as buffered saline introduced by syringe to wash out a length of the intestine (Gorbach *et al.*, 1967b). Samples of intestinal contents can be obtained through a peroral or nasal tube. This technique has disadvantages apart from discomfort, the tube may stimulate the intestine causing gastric and billary secretion, thus changing the intestinal contents.

1.2.4.3 Problems Of Quantification

Two methods can be used to quantify microorganisms in a sample; 1) the total microscopic count AND 2) the viable count of the most numerically important groups of microbes, here detection of the microflora is carried out by serial dilutions of samples plated on selective and/or differential media as well as non-selective media (which can be either pre-reduced or reduced) and incubated in both anaerobic and conventional environments. Individual colonies are then selected and identified.

A search for alternative methods for detection must be made, as it is known that not all intestinal organisms are isolated and as a consequence identified by the methods available. Many of the organisms present have fastidious growth requirements and require selective or differential media for isolation, hence a failure to use selective and differential media will result in failure to detect a number of organisms present in counts lower than the numerically dominant flora. An advantage of these selective techniques is that they can detect bacterial species present in low counts which may be missed by other methods. It also permits ready differentiation of various colony types on a variety of media, but is limited by the availability of differential selective media.

Both methods for counting have inherent inaccuracies in addition to those arising from the dilution series (Meynell & Meynell, 1970). The total viable count made on a non-selective solid medium estimates the number of colony forming units (CFU) per gram sample. Since each colony can arise from one cell, a clump, or a chain of cells, it is important to count microscopically in the same manner. Comparison of the results from using the two methods normally has the consequence that the total viable count is less than the total microscopic count. This could be due to a proportion of dead organisms in the sample (Clarke, 1977).

Microbial activity can be used as an alternative method to quantify numbers of organisms present by the application of microbiological assays (Humble *et al.*, 1977; Molly *et al.*, 1993). Thus, the estimation of faecal β-gluconidase of bacterial origin has been suggested as a more sensitive indicator of the influence of diet on the flora than counting bacterial species. In the intestine, microbial activity can be measured directly by the measurement of products of bacterial degradation, that is metabolic activity (Macfarlane *et al.*, 1992), by studies into the degradation products of bile acids and pigments.

1.2.4.4 Culture Of Intestinal Samples

Aerobes and facultative anaerobes can be cultured on commercially available selective media, although these are inhibitory and even reduce the apparent count of the organisms for which they are selective, however they do allow for partial enumeration of easily recognisable groups, such as coliforms, *Staphylococci* and *Streptococci.*

Reliable methods for the cultivation of intestinal flora involves exclusion of oxygen by the use of the roll tube method (Hungate, 1969) or an anaerobic chamber (Finegold, 1969; Drasar & Crowther, 1971; Finegold *et al*, 1983). Both systems have advantages and disadvantages, related to ease of operation and cost but both produce consistent viable counts.

1.3 Intestinal Pathogens

The indigenous microflora of a given body site, being an ecosystem in the climax stage, is stable in its composition. Stability implies that "invading" microbes, that is, those that enter the site from the environment are prevented from colonizing it. If the "invader" is a pathogen, this effect constitutes a potent host defence mechanism (Zubrycki & Spaulding, 1962; Aly & Shinefield, 1982; Freter *et al*, 1983).

In UK and other developed countries, infections of the human digestive tract are most commonly associated with infectious (pathogenic) bacteria present in food. The symptoms that arise from consumption of contaminated food are well known, including abdominal pain, diarrhoea, nausea and headache.

Data from the Office of Population Census & Surveys (OPCS Monitor) and Communicable Disease Report (CDS Report) has shown the total number of notified cases of food poisoning (including cases ascertained by other means, Sockett *et al*, 1993) has increased steadily from 1985 to December 1993 (See

Table 1.3a).

Table 1.3a Formal Notifications Of Cases Of Food Poisoning (1985-1993)

YEAR	REPORTS OF FOOD POISONING IN UK.
1985	14,500
1986	20,050
1989	31,488
1991	35,574
1992	42,241
1993	53,763

It is disturbing to note that the annual total of reported cases of food poisoning continues to increase. However, it is believed that the true number of actual cases is ten times the number of cases actually reported, mainly because the symptoms of the patient are not deemed serious enough to seek medical attention. It should be noted that these figures represent the number of cases where the causative organism has been identified and the actual number of cases of food poisoning reported where the causative organism has not been identified is approximately 2.5 times greater than those represented in Table 1.3a.

Foodborne infections cause financial burden on society with respect to healthcare costs and loss of earnings. It has been estimated that cost of foodborne infections to the NHS runs to millions of pounds each year and is responsible for the loss of more than 23,000,000 working days each year. There are two types of food poisoning organisms: A) INVASION those in which the food serves merely as a carrier for the inoculum which then grows in the consumer, such as *Salmonella spp.* These require only low cell dosage (100cells/g) for an effective dose dependent upon the amount of food consumed AND B) INTOXICATION others in which the organisms grow in the food and produce toxins which then affect the consumer soon after consumption, such as *Staphylococcus aureus*, *Clostridium botulinum* and *Bacillus cereus* where high cell dosages (10^6/g) are required for an effective dose. The toxins produced by bacteria can be divided into two groups; 1) those liberated from multiplying bacteria (exotoxins) AND 2) those associated with the cell envelope which are released after the death of the bacterium (endotoxins).

There is some overlap between these categories as some organisms (for example *Shigella* or *Salmonella*) may have invasive properties as well as being able to produce toxin. The pathogenic bacteria commonly associated with intestinal disease include *Campylobacter spp*, *Shigella dysenteriae*, Enteropathogenic *E. coli*, *Clostridium spp*, *Yersinia spp*, *Vibrio cholera* and *Listeria monocytogenes.*

The number of reported cases of food poisoning and associated diseases in 1993 where the causative organism was identified is shown in Table 1.3b.

Table 1.3b Reported Cases Of Food Poisoning In 1993

LABORATORY REPORTS FOR 1993	CUMULATIVE TOTAL
Campylobacter spp	39,383
Shigella spp	6,904
Salmonella spp	737
Enteropathogenic E. coli	430
Clostridium difficile	5,246
Yersinia spp	358
Vibrio spp	86
Listeria monocytogenes	69

1.3.1 *Campylobacter spp*

For enteritis and associated diseases, laboratory reports in England and Wales showed a remarkable increase from 1,349 cases in 1977 to more than 38,000 in 1992. This is significant when one considers the cost of treatment in 1989 for this organism alone was £9 million, with a cost per patient of £273 (Skirrow, 1990). The pathogenesis of *Campylobacter jejuni* gastrointestinal disease is not fully understood, however it is characterised by destruction of the mucosal surfaces of the jejunum, ileum and colon. *Campylobacter pyloridis* in the human stomach makes its way through the mucus by corkscrew movements, and may cause gastritis. As of yet no enterotoxin has been detected and it seems likely that the bacteria invade mucosal cells (Hazell *et al.*, 1986). *Campylobacter* has been isolated from raw meat and poultry as well as unpasteurised milk.

1.3.2 *Shigella dysenteriae*

Shigella dysenteriae attaches specifically to colonic epithelium and enters the cell after locally dissolving the cell membrane, this leads to focal mucosal ulcers through which erythrocytes and white blood cells exude to produce the typical dysenteric stool. There is great variation in virulence within Shigellas, *Shig. dysenteriae* type 1 causes severe dysentery due to the production of a cytotoxin known as Shiga toxin, *Shig. sonnei* is as invasive as *Shig. dysenteriae* type 1 using the same mechanism but rarely causes the dysentery syndrome. Some strains of pathogenic *E. coli* (such as strain 0124), behave in a similar manner to the shigellas.

1.3.3 *Escherichia coli*

Certain strains such as ETEC produce a similar and antigenically related enterotoxin which attaches to different receptors in the intestinal epithelium. These strains make specific attachments enabling them to colonize intestinal epithelium so that the enterotoxin can be absorbed and act locally. Some produce additional enterotoxin that activates guanylate cyclase, contributing further fluid loss and pathogenicity (Field, 1979). The annual number of infections due to verocytotoxin producing strains of *E. coli* 0157, which cause haemorrhagic colitis and HUS, more than doubled during 1989 and 1991 (CDSC, 1991). Some strains of pathogenic intestinal bacteria penetrate the intestinal epithelial cells after attachment.

1.3.4 *Clostridium spp*

Clostridium difficile can produce 2 types of toxin (A and B). Type A has been referred to as the enterotoxin because it causes fluid accumulation in the bowel, but the mechanism of action is not through stimulation of adenyl cyclase. Type B toxin does not cause fluid accumulation but has been shown to be cytopathic for all cell tissue cultured cells tested. *Clostridium perfringens* food poisoning in humans is produced by type A strains, the enterotoxin is a product of the sporulation process and causes fluid accumulation in the small bowel.

1.3.5 *Yersinia spp*

The term yersiniosis suggests a specific syndrome similar to shigellosis, however human infections caused by *Y. enterocolitica* and *Y. pseudotuberculosis* vary from severe septicaemic-type illness similar to typhoid fever through to mild enterocolitic infections. Although the pathogenicity of the organism has not been finally established, there is evidence to suggest a relationship between its presence and the symptoms of disease, it is known that *Yersiniae* multiply in the gut mucosa particularly the ileum. It has been isolated from patients with acute terminal ileitis, mesenteric adenitis, appendicitis and erythema nodsum, as well as from those with mild and severe gastroenteritis. Recently such organisms are being suspected of association with Crohn's disease.

1.3.6 *Vibrio cholera*

Vibrio cholera multiplies in the lumen of the small intestine and does not invade tissues. Helped by its motility it attaches to intestinal epithelium and releases a toxin that reacts rapidly with receptors on epithelial cells and makes a specific attachment. It does not damage the cell but activates adenylate cyclase and thereby raises the intracellular level of cyclic adenosine monophosphate (cAMP). As a result, water and electrolytes are lost through the intact epithelial cells into the small intestine. As the multiplying bacteria increase in number and more cells are affected, the absorptive capacity of the colon is overwhelmed and there is a profuse watery diarrhoea (Cash *et al*, 1974).

1.3.7 *Listeria monocytogenes*

In mid 1989 the increase in human listeriosis and its association with contaminated food (due to living cells present in meat, poultry, soft ripened cheeses, pate and raw vegetables) generated public concern and evidence has accumulated of the widespread distribution of listeriosis, especially in certain European countries and in North America (WHO, 1988; Jones, 1990; Newton *et al*, 1990). In England

and Wales reported cases ranged around 20/year, however no outbreaks of foodborne listeriosis were recorded from 1989-91. It manifests clinically, in older adults, as a meningoencephalitis but a generalised infection (granulomatosis infantiseptica) may occur in neonates. Inapparent infection may occur at any age, in pregnant woman it may be followed by abortion, usually in the 5th-6th month. It is known that *Listeria* produces an enterotoxin in the form of a haemolysin which probably plays little part in pathogenicity. Although the numbers of reported cases is low, *Listeria* is of particular concern due to the high percentage incidence of fatalities.

1.3.8 *Salmonella spp*

These pathogens not only penetrate epithelial cells, but also invades subepithelial tissues, sometimes multiplying in the phagocytes that engulf them. In man, this group includes *Salmonella typhi* and *paratyphi*, which invade and cause a characteristic systemic disease (typhoid or enteric fever) (Middlebrook & Dorland, 1984; Baird-Parker, 1990). In 1988/89, the cost of Salmonella infection was estimated at £231-331 million annually (Sockett & Roberts, 1991).

In view of the inherent cost of foodborne/intestinal related disease to society and concern about the ever-increasing incidence rate, investigations into how the incidence of foodborne infection can be controlled and or prevented are necessary.

1.4 Present Investigation

Realisation that dietary modification for the prevention of disease would be more effective and economic in large populations than costly drug treatment and modern medical equipment, has stimulated an interest in the effects of various dietary components including vegetables, herbs and spices. Garlic offers a mild safe option for the self-treatment of a number of chronic or repeating and self-limiting, non-serious infections. These include those of the mouth (gum infections), throat (tonsillitis), upper respiratory tract (bronchitis, cough, catarrah), digestive system

(food poisoning), urogenital system (cystitis), fungal infections of the skin and candidal infections of the mucous membranes. The UK product licence for garlic allows the claim of a herbal remedy traditionally used in the treatment of colds, coughs, rhinitis and catarrah.

In view of the significantly incomplete knowledge on the effect of garlic oil on bacteria and the current concern with the level of food poisoning, the major aims of this investigation were:-

A) to study the antimicrobial nature of both a steam distillate of garlic containing dialk(en)yl sulphides (garlic oil) and a freeze-dried garlic powder generating predominantly thiosulphinates.

B) to develop model systems in order to assess the impact of garlic preparations and their components on mixed microbial communities under gut-simulated conditions.

C) quantitative analysis of both garlic products, in order to identify components and monitor changes of garlic preparations during antimicrobial studies.

D) study of the antimicrobial mode of action of the garlic materials investigated.

CHAPTER 2

METHODS & MATERIALS

2.0 METHODS & MATERIALS

2.1 METHODS

2.1.1 Preparation Of The Garlic Products

The following procedures were employed to prepare standardised sterile preparations of the garlic products prior to any subsequent microbiological studies.

2.1.1.1 Freeze-Dried Garlic Powder (G.P)

An initial 10% (w/v) suspension of garlic powder was prepared with double distilled water. The preparation was then agitated vigorously by hand for 1 minute, then left for 30 minutes at room temperature (to allow for complete enzymatic conversion of alliin to allicin). After this standing period, the preparation was centrifuged at 10,000 rpm in the MSE 18 at 4°C for 15 mins and the supernatent collected. The extract was then sterilised using a positive pressure filter sterilisation method (through a glass fibre filter 0.4µm) in order to remove bacteria known to be present in the powder.

2.1.1.2 Steam Distilled Garlic Oil (G.O)

No prior preparation of the garlic oil was performed.

2.1.2 Demonstration Of The Antimicrobial Activity Of The Garlic Products

2.1.2.1 Minimal Inhibitory Concentration Test (MIC)

The MIC test determines the lowest concentration of G.O/G.P, which is required to inhibit the growth of a microbe *in vitro.*

A series of 16ml volume test tubes with serial two-fold dilutions of the garlic products in a broth medium, TSB (Tryptone Soy Broth, Difco) were prepared (final volume of 5ml). Each tube was inoculated with one drop of an overnight culture of the organism under investigation (approximate cell density of $5x10^6$ cells/ml), so that no visible turbidity was imparted to the broth. The tubes were then incubated at 37°C for upto

48 hours and the MIC recorded as the lowest concentration showing complete inhibition of visible growth.

2.1.2.1.1 The Effect Of G.O Solubility On G.O Antimicrobial Activity

10% dilutions of G.O in TSB were prepared in universal bottles, the contents were shaken to produce homogenous suspensions. From this serial two-fold dilutions of the homogenous mixture were prepared in TSB, in order to perform MIC determinations with *E. aerogenes* (3) (See 2.1.2.1). The remaining homogenous suspension was allowed to settle for 20 minutes and formed two distinct fractions. MIC determinations were then performed on the top and bottom fraction as well as the interface between the two.

2.1.2.2 The Effect Of Garlic Products On Cell Viability

Serial two-fold dilutions of the garlic products in TSB (final volume of 20ml) were prepared in capped 50ml boiling tubes. The tubes were inoculated with 40µl of an overnight culture of the organism under investigation and the viable cell numbers determined with respect to time by either the spread plate or Miles and Misra techniques. The results were compared to a control of the organism in TSB without garlic product added.

Viability studies involving *Lactobacillus acidophilus* NCFM (Gilliland Strain) in pure or mixed culture were performed in deMann Rogosa Sharpe broth (MRS), due to the inability of *L. acidophilus* to grow in TSB. The composition of MRS broth was as follows:

	g/L
Beef Extract	10.00
Yeast Extract	10.00
Peptone	5.00
Glucose	20.00

Tween 80	1ml
K_2HPO_4	2.00
Sodium Acetate	5.00
Tri ammonium Citrate	2.00
$MnSO_4.4H_2O$	0.20
$MgSO_4.7H_2O$	0.20

The components were dissolved in 800ml of double distilled water and then made up to 1 litre. MRS broth was then sterilised at 15lbs/in^2 for 15mins.

The relationship between cell death/growth rate and concentration of garlic product was calculated by taking the initial steepest slope and incorporating the following formula:-

$$\text{DEATH/GROWTH RATE} = \frac{(\text{Log } b - \text{Log } a)}{\text{Log } 2 \times t}$$

Where b = viable cell population at time t_2

a = viable cell population at time t_1

t = time difference (in minutes), $t_2 - t_1$

2.1.3 Determination Of Volatilisation Of Antimicrobial Garlic Compounds

The following experiments were designed to provide evidence to support the theory of volatilisation.

2.1.3.1 The Effect Of Agitation By Forced Aeration/Nitrogenation

10% (v/v) suspensions of G.O in TSB (final volume of 30ml) were prepared in 50ml boiling tubes and agitated by forced aeration/nitrogenation by the passage of sterile air or nitrogen (flow rate of 200cc/min) through the suspension. Loss of antimicrobial activity was determined by; a) Time-dependent MIC determinations (See Section 2.1.2.2) AND b) High Performance Liquid Chromatography (HPLC) analyses (See

Section 2.1.6.3).

2.1.3.2 The Effect Of Temperature/Agitation; The Effect Of Air-Tight Sealing Of Flasks

10% (v/v) suspensions of G.O in TSB (10ml volume) were prepared and pre-incubated statically at 37°C or at 37°C in a shaking water bath for 24 hours. After incubation the samples were assessed for their antimicrobial activity by incorporating the samples into the MIC method. The experiment was repeated in sealed air-tight flasks to investigate the loss of antimicrobial components to the atmosphere.

2.1.3.3 The Effect Of Environmental Conditions Upon G.O Activity

The following non-growth supporting and growth-supporting media were assessed: 25mM and 50mM Potassium Phosphate Buffer at a pH of 7.0; 0.5M Sodium Bicarbonate; Double Distilled Water; 0.9% Saline; Minimal Salts Medium (MSM) (composition of which in gL^{-1} was; KH_2PO_4 1.0, K_2HPO_4 1.0, KNO_3 1.0, $(NH_4)_2SO_4$ 1.0, $MgSO_4.7H_2O$ 0.1, NaCl 0.1) with and without addition of glucose at a concentration of 1.0%; Tryptone in MSM at a concentration range of 5-15g/L and L-cysteine in MSM at a concentration range of 1-25mM.

A 2.75mg/ml G.O concentration was prepared in each of these media (final volume of 20ml) in tin foil capped 50ml boiling tubes. 40µl of an overnight culture of *E. aerogenes* (3) was used as an inoculum. The tubes were then incubated in a water bath at 37°C and the cell viability monitored with respect to time as described in Section 2.1.2.2. Control experiments employing the same media without G.O addition were also set up.

2.1.4 Evaluation Of Potential Antimicrobial Effects Of G.O Within The Gut Environment

In order to proceed with this type of work initial modelling procedures using synthetic gut fluids were employed before advancing to the use of 'real' gut fluids.

2.1.4.1 Synthetic Gut Fluid Models

Two synthetic gut fluids were used for initial analyses of the antimicrobial activities of G.O.

2.1.4.1.1 Simple Simulated Intestinal Fluid (UPS XX1, 1985), SIF.

The composition of this fluid is as follows:

		g/L
Monobasic Potassium Phosphate		6.8
Distilled Water	250ml	
add to 0.2N Sodium Hydroxide	190ml	
Distilled water	400ml	
Pancreatin		10.0

mix, adjust pH to 7.5 with 0.2N NaOH

make up with distilled water to 1L.

Sterilisation was achieved by positive pressure filter sterilisation through a 0.4µm glass microfibre filter into sterile plastic universal bottles. Alternatively sterilisation was achieved by autoclaving the whole synthetic fluid at 15lbs^2 pressure for 15 mins at 121.6°C.

2.1.4.1.2 Complex Simulated Intestinal Fluid (Duodenum Medium), CIF.

The composition of CIF was as follows:

	g/L
1. D-glucose	24.00
2. Casein-peptone	5.70
3. $NaHCO_3$	1.00
4. NaCl	6.14
5. KH_2PO_4	0.68
6. NaH_2PO_4	0.30
7. Porcine-Bile Extract	5.60
8. Lysozyme	0.60
9. α-amylase	1200 U/L
10. Lipase	1000 U/L
11. Trypsin	110 U/L
12. α-chemotrypsin	350 U/L
13. Resazurin	0.002

Components 2, 3, 4, 5, 6 and 13 were dissolved in 850-900ml distilled water, the pH adjusted to 6 with 8N NaOH or 8N HCl and sterilized for 10 mins at 115°C. The glucose was dissolved in 100ml distilled water and sterilized for 10 mins at 115°C. While both solutions were still hot the glucose solution was aseptically added. Concentrated stock-solutions of components 7, 8, 9, 10, 11 and 12 were added after being filter sterilised through a swinnex filter.

2.1.4.2 Antimicrobial Activity Of G.O Within Synthetic Fluids

Pure culture experiments were initially performed in order to provide some comparison to antimicrobial activity of garlic oil in TSB (Section 2.1.2.1 & 2.1.2.2). These included MIC determinations and cell viability studies using *E. coli* (40), *E. aerogenes* (3), *L. monocytogenes* (433), *S. typhimurium* (434) and *Shig. sonnei* (426).

Simple mixed culture experiments were then performed with *E. coli*, and *L. monocytogenes.* These organisms were chosen due to; 1) differences in sensitivity towards the G.O AND 2) ease of identification of *L. monocytogenes* on Listeria Selective Agar (LSA), Difco (See Appendix, Plates 1-8).

2.1.4.3 Real Gut Fluid Models (Ileostomy Samples, IF)

Ileostomy effluents were obtained from patients having a section of the ileum (the third portion of the small intestine) removed due to either inflammatory bowel disease, ulcerative colitis, Crohn's disease or familial adenomatous polyposis.

The operation involves the establishment of a fistula known as the "stoma" following the removal of the whole of the large bowel, rectum and anus, the end of the terminal ileum is brought through the abdominal wall, everted to form a spout, and sutured to the skin surface. The body's effluent is discharged through the ileostomy into a collecting bag adhered to the skin, which can be emptied at intervals. It should be noted that there are a number of types of ileostomy operation:-

A) temporary loop ileostomy - allows 'resting' of the whole colon. It is used to protect ileorectal and ileoanal anastomoses, a loop of ileum is brought to the surface of the body and supported on rubber tubing or a short rod. After the ileum has been cut round two thirds of its circumfurance on one side of the loop, a spout can be formed from the longer arm of the loop.

B) terminal ileostomy - is made when the entire colon needs to be removed, this occurs most often in inflammatory bowel disease, but may also be necessary in familial polyposiscoli and very occasionally in cases of colorectal cancer. The ileum is usually divided about 2cm in front of its junction to the caecum. The ileum is then brought out through a 2cm incision in the rectus muscle to a length of about 6-7cm. It is sutured to the abdominal wall to prevent it retracting, then turned 'inside out' (everted),

to form a spout of 2-3cm in length. This spout is necessary to keep the extremely irritant ileal fluid off the sensitive skin.

Two types of IF samples were collected; 1) from patients on the surgery ward at Queen Elizabeth Hospital, Birmingham, having had ileostomy operations 4-8 days prior to sampling AND 2) from asymptomatic out-patients at West Park Hospital, Wolverhampton, having long established, well functioning ileostomies (1-10 years post-operation). All were between 20 to 50 years of age and had undergone total colectomy for relief of idiopathic ulcerative colitis (except 1, who had Crohns disease).

The ileostomy discharges were allowed to flow directly from the ileostomy bag into sterile 250ml Schott bottles, stored on ice during transportation. All experimental studies were initiated within 1 hour after the specimens were collected. In order to determine the degree of variability in numbers of microorganisms, weekly samples were collected from the patients of West Park Hospital over 4 weeks.

After initial enumeration of the collected individual ileostomy samples, samples were pooled to provide sufficient fluid for experimental use.

2.1.4.3.1 Enumeration And Identification Of The Microorganisms Present In IF

Total viable counts were performed by ten-fold serial dilutions of individual IF samples in sterile ¼ strength ringer solution. 100µl of each dilution was then spread onto a variety of differential and selective media which included; Difco Tryptone Soy Agar (TSA), LabM MacConkeys Agar with salt (MC), LabM MacConkeys Agar without salt (MCNS), TSA plus 5% Gibco defibrinated Horse Blood Serum (BA), Difco Clostridium Agar (CA), Difco Tomato Juice Agar (TJA), Difco Brain Heart Infusion (BHI). Triplicate plates were incubated at 37°C under anaerobic and aerobic conditions for upto 48 hours. Individual colonies were then streaked onto fresh agar plates to provide a stock of pure cultures of microorganisms isolated (See Appendix, Plates 9-

14). The range of media was used to determine the most suitable types to be used in subsequent experiments. Isolated microorganisms were tentatively identified using the Grams staining technique and API biochemical strips.

1. Grams Staining Technique

a) Heat fix a smear of the unidentified colony

b) Flood the slide with crystal violet for 1 minute

c) Flood with Grams iodine for 2 minutes

d) Drain with ethanol for 10 seconds, immediately rinse with distilled water

e) Flood with safranin for 1 minute

Identification was carried out by comparison of colonial morphology on different media and Gram stain reaction to information in Bergey's "Manual of Determinative Bacteriology".

2. API Biochemical Strips

Commercially available plastic strips containing a variety of biochemical tests relevant to specific groups of microorganisms: API 20E - for enteric bacteria, API 20NE - for non enteric bacteria, API STAPH - for *Staphylococcus spp.*, were employed.

Each tube on the strip was inoculated with one drop of an overnight culture of the organism to be identified, the strips were then incubated at the relevant temperature for the designated period of time. The results of individual tests where then processed using the API computer programme. Data from computer analysis provided a 90-99% positive identification of certain microorganisms present in the IF.

2.1.4.3.2 Antimicrobial Activity Of G.O Within IF

In order to observe the effect of IF on the antimicrobial activity of G.O and to provide pure culture data comparable to that obtained with TSB, SIF and CIF, attempts were made to sterilise ileostomy fluid.

The heavy consistency of IF is such that positive pressure filter sterilisation was not suitable. Autoclaving the IF at 15lbs/in^2 pressure for 15 minutes at 121.6°C removed all the microorganisms present in the fluid, however alteration to its biochemical composition was also deemed likely. Therefore centrifugation of IF at 14,000 rpm at 4°C for 30 minutes was used as an alternative to sterilisation as it resulted in a hundred-fold reduction in cell population numbers. Sonication of IF samples on ice for 10 minutes at the settings used to emulsify crude oil, was also found to be partially effective for complete sterilisation of the fluid. As none of the methods performed were ideal it was decided to work primarily with complete whole IF samples.

2.1.4.3.2.1 The Effect Of Various Concentrations Of G.O On The Microflora Present In IF Inoculated With *Listeria monocytogenes* (433)

Serial two-fold dilutions of G.O in IF were prepared in capped test tubes (final volume of 10ml). The tubes were inoculated with 20µl of an overnight culture of *L. monocytogenes* and the viable cell numbers determined with respect to time by the spread plate technique for the bacteria present in IF onto TSA, MC and BA plates and onto LSA. The results were compared to a control, to which no G.O was added.

2.1.5 Indirect Determination Of The Allicin Content Of Garlic Powder Preparations

The determination of allicin relies on the enzymatic conversion of alliin (catalysed by alliinase) to allicin, pyruvate and ammonia. The allicin content was indirectly determined by measuring the concentration of the end products of the reaction, pyruvate or

ammonia.

Garlic power extracts were prepared according to 2.1.1.1 and immediately subjected to analysis for pyruvate and ammonia. Pyruvate was determined spectrophotometrically using Lactate dehydrogenase (LDH) and NADH based upon the assay systems of Schwimmer & Mazelis (1963); Kazaryan & Goryachenkova (1979), while ammonia was determined spectrophotometrically by the catalysed indophenol colour reaction based upon the assay system of Chaney & Marbach (1962). The formation of both end products was measured using a Cecil 599 Spectrophotometer at the wavelengths of 630nm (ammonia) and 340nm (pyruvate) respectively.

2.1.6 Quantitative Analysis Of Garlic Products By Gas Chromatography And High Performance Liquid Chromatograpy

2.1.6.1 Gas Chromatographic Analysis Of Dialk(en)ylsulphides From Steam Distilled Garlic Oil

10-15mg of G.O was extracted with 5ml of Isooctane. 1mg of the internal standard dipropyl disulphide (of 99% purity) was added for a quantitative estimation. 0.2µl of the sample was then directly injected into the GLC.

2.1.6.1.1 GLC Conditions

A Perkin-Elmer 8420 GC with a DB wax capillary column (30x0.32mm, film thickness) was used for the analysis. This column is a polar type and used for the analysis as it can achieve both a high resolution and good base line separation (Yan *et al*, 1992; 1993. The carrier gas was Helium at 22.5 psi and cold on-column injection was used.

The temperature programme was set as follows:

$$55^{\circ}C\ (10min) \xrightarrow[\text{ramp rate}]{4^{\circ}C/min} 150^{\circ}C \xrightarrow[\text{ramp rate}]{15^{\circ}C/min} 220^{\circ}C\ (10min)$$

Note - 10 minutes isothermal at 55°C at the beginning of the analysis is essential in order to facilitate the separation of the internal standard (dipropyl disulphide) from dimethyl trisulphide.

2.1.6.2 Gas Chromatographic Analysis Of Vinyldithiins From Freeze-Dried Garlic Powder

1g of freeze-dried G.P was homogenised with 30ml water using an Ystral homogeniser for 1 minute on ice. The homogenate was then stood for 30 mins at room temperature to allow alliin conversion to allicin. This was then centrifuged at 10,000 rpm (MSE Europa M24) for 15 mins at 4°C. 2ml of the supernatant was then applied to a preconditioned C_{18} (SEPPAK) cartridge and the allicin eluted with 15ml of isooctane. Any water present in the eluate is removed from the sample before injection by adding sodium sulphate crystals. The isooctane eluate was then heated to 100°C in a gas tight vial for 10 mins using a heating block (in order that allicin converted to vinyldithiins). 2mg of dipropyl disulphide (internal standard) was then added to the eluate. 0.2µl of the sample was then directly injected into the GLC.

Note - The SEPPAK C_{18} cartridge must be conditioned before use, by passing 2ml methanol and then 5ml water through it.

2.1.6.2.1 GLC Conditions

The GLC conditions employed were identical to those for the separation of dialk(en)yl sulphides (Section 2.1.6.1.1).

2.1.6.2.2 Peak Identification

Due to the unavailability of pure standards, identification of the vinyldithiins was carried out by comparison of the retention times of samples previously examined by Yan *et al.* at Humberside University.

2.1.6.3 High Performance Liquid Chromatographic Analysis Of Dialk(en)yl sulphides From Steam Distilled Garlic Oil

10mg of G.O (or individual sulphides) was extracted with 10ml of acetonitrile. The acetonitrile extracts were then filtered through an HPLC filter and 5µl of the extract directly injected into the HPLC. In order to determine changes in G.O composition during antimicrobial studies, equivalent volumes of acetonitrile were added to the concentration of G.O in TSB. The extracts were then filtered through an HPLC filter and 5µl of the extract directly injected into the HPLC system for detection.

2.1.6.3.1 HPLC Conditions

Analyses were carried out using a Gilson HPLC system with a PYE UNICAM PU4020 UV Detector (Phillips) and Trio Integrator (at Humberside University) and by a LKB Bromma HPLC system with a 2152 Controller, 2150 HPLC Pump, 2158 UVICORD SD and 2220 Recording Integrator (at University of Wolverhampton). A spherisorb ODS-2 column with a flow rate of 1ml/min, ACN:H_2O:THF, (ACETONITRILE: WATER: TETRAHYDROFURAN) (70:27:3) was used as mobile phase, with the UV detector set at 254nm.

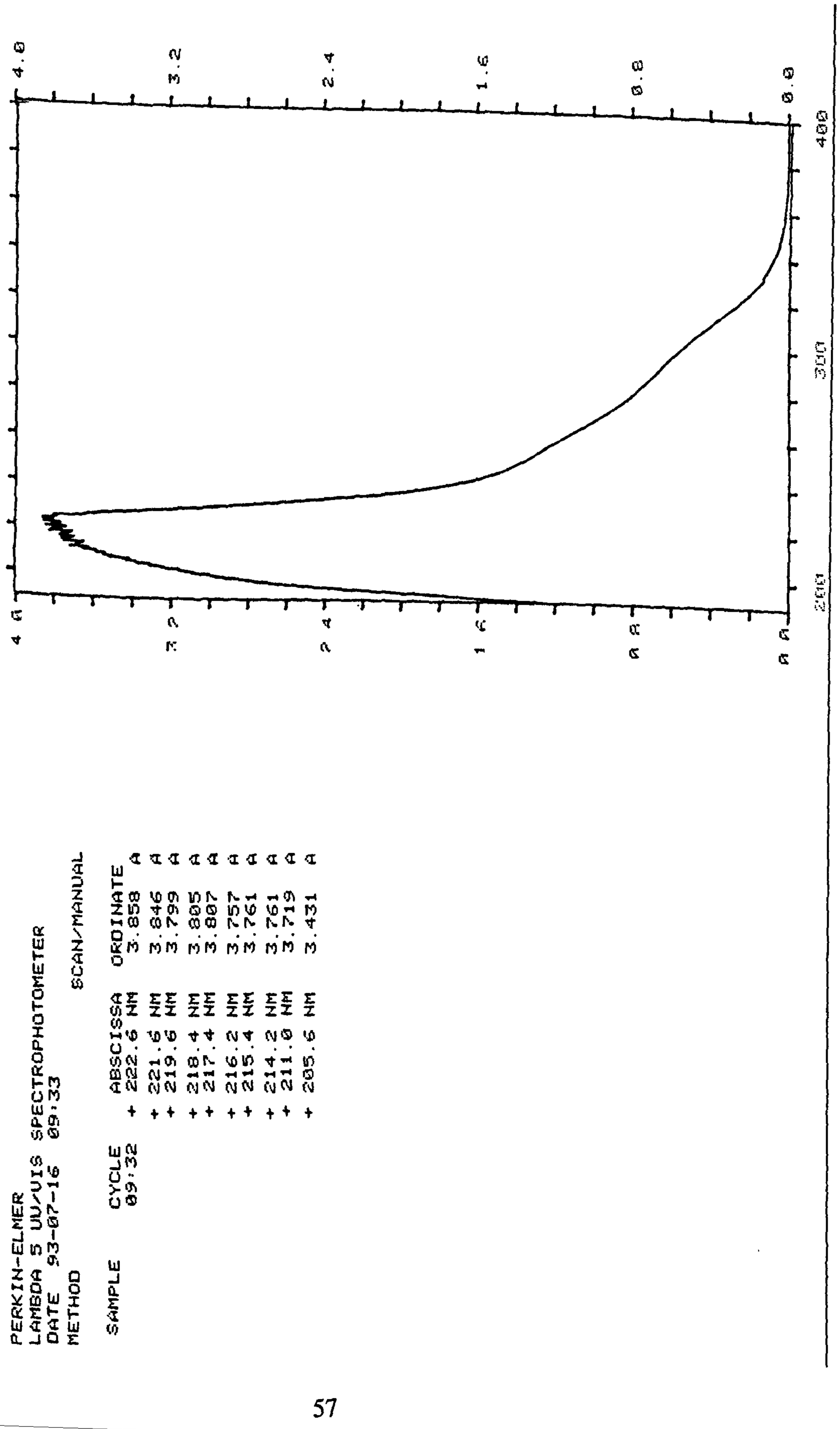
In order to try to improve the method of HPLC analysis with respect to baseline/ baseline separation of the individual sulphide peaks, initial changes to the conditions of analysis were performed. Alteration of the flow rate from 1.0ml/min to 0.5ml/min, change of mobile phase to 50:50 (ACN:H_2O) and the use of a gradient system however did not result in an improvement of the sensitivity (in terms of baseline/baseline separation) or the sharpness of the peaks.

HPLC analyses were initially carried out at a detector wavelength of 254nm as were those by Lawson (Personal Communication). However a UV spectrum of G.O, at the settings given in Figure 2.1.6.3.1a, showed that a wavelength of approximately 230nm gave the greatest absorption for G.O.

Figure 2.1.6.3.1.a: UV Spectra of G.O

PERKIN-ELMER	
LAMBDA 5 UV/VIS SPECTROPHOTOMETER	
DATE 93-07-16 09:34	
SAMPLE ID ..... G.O SAMPLE /ACN VS ACN	
OPERATOR ..... ZARA	
METHOD	SCAN / MANUAL
01 ORDINATE MODE	ABS
02 SLIT	2 NM
03 SCAN SPEED	240 NM/MIN
04 RESPONSE	0.2 S
05 LAMP	332.8 NM
06 CYCLES/TIME	1 / 0.05 MIN
07 PEAK THRESHOLD	0.02 A
08 RECORDER	SERIAL / DASH 1
09 ORD MIN/MAX	0.000 / 4.000
10 ABSC MIN/MAX	190.0 / 400.0
11 ABSCISSA FORMAT	20 NM/CM
12 PRINTER	GRID/SCALE/SCREEN/METHOD

Figure 2.1.6.3.1a



HPLC analysis of G.O was then performed with the detector set at a wavelength of 230nm and compared to that determined at 254nm. The results (Table 2.1.6.3.1b) indicated that changing the UV detector wavelength from 254nm to 230nm resulted in greater absorption peak areas and hence gave a greater sensitivity of detection of virtually all G.O sulphide components. Two-fold and even a three-fold increases in peak areas were obtained for some of the G.O components. Peak areas of sulphides containing allyl groups exhibited the greatest increase in absorption when switching the detector wavelength from 254nm to 230nm, whereas DMT showed only a small increase and DMD a slight decrease in peak area at 230nm. This overall effect is attributed to the increased molar absorbtivity of some sulphides at 230nm than at 254nm. However it should be noted that analyses at 230nm were not used for further studies due to insufficient time.

Table 2.1.6.3.1b Differences In Peak Area Using Detector Wavelengths Of 230 & 254nm

		Peak Areas	
Retention Times	Sulphides	254 nm	230 nm
4.13	(DMD)	132572	113867
4.65	(DAS)	256924	638436
4.98	(DMT)	155139	247235
5.30	(DADS)	601771	1676324
5.68	(MATS)	912751	1837963
6.59	(DATS)	848471	1991339
7.07	(MATTS)	579302	1118833
8.38	(DATTS)	735556	1672498

Qualitative and relative quantitative analyses results for G.O components presented in this thesis were determined using the HPLC system with respect to the UV detector set at 254nm. It is recommended that future analyses be performed with the HPLC detector set at 230nm subject to further investigation into determining the absence of any interfering compounds potentially present in the garlic oil or experimental media.

In order to facilitate relative quantification of sulphide components between samples, calibration graphs (Figures 2.1.6.3.1c, d) of individual sulphide peak area versus concentration of G.O were determined. The linearity of the individual component peak areas with respect to G.O concentration was assessed by linear regression analysis and for each of the sulphides the coefficient was greater than 0.99% for G.O concentration in the range of 0.0625-1.5mg/ml.

The reproducibility of the results was determined by five replicate sample injections (of each G.O concentration) into the HPLC system, using 20µl sample volumes. The % coefficient of variation (%CV) was determined for all individual sulphide components of the G.O and found to lie within the 5% error range.

Figure 2.1.6.3.1c: Analysis Of HPLC Linearity For G.O Sulphides (DMD, DMT, MATS & MATTS)

LEGEND:

●	DMD	———	LOBF	▲	DMT	———	LOBF
■	MATS	———	LOBF	◆	MATTS	———	LOBF

Figure 2.1.6.3.1d: Analysis Of HPLC Linearity For G.O Sulphides (DAS, DADS, DATS & DATTS)

LEGEND:

○	DAS	———	LOBF	△	DADS	———	LOBF
□	DATS	———	LOBF	◇	DATTS	———	LOBF

Calibration curves of peak area vs concentration of G.O injected into the system have been determined in order to calculate the linearity of this HPLC analysis. Linear regression analysis was performed on all peak areas obtained in order to determine the line of best fit (LOBF).

Figure 2.1.6.3.1 c

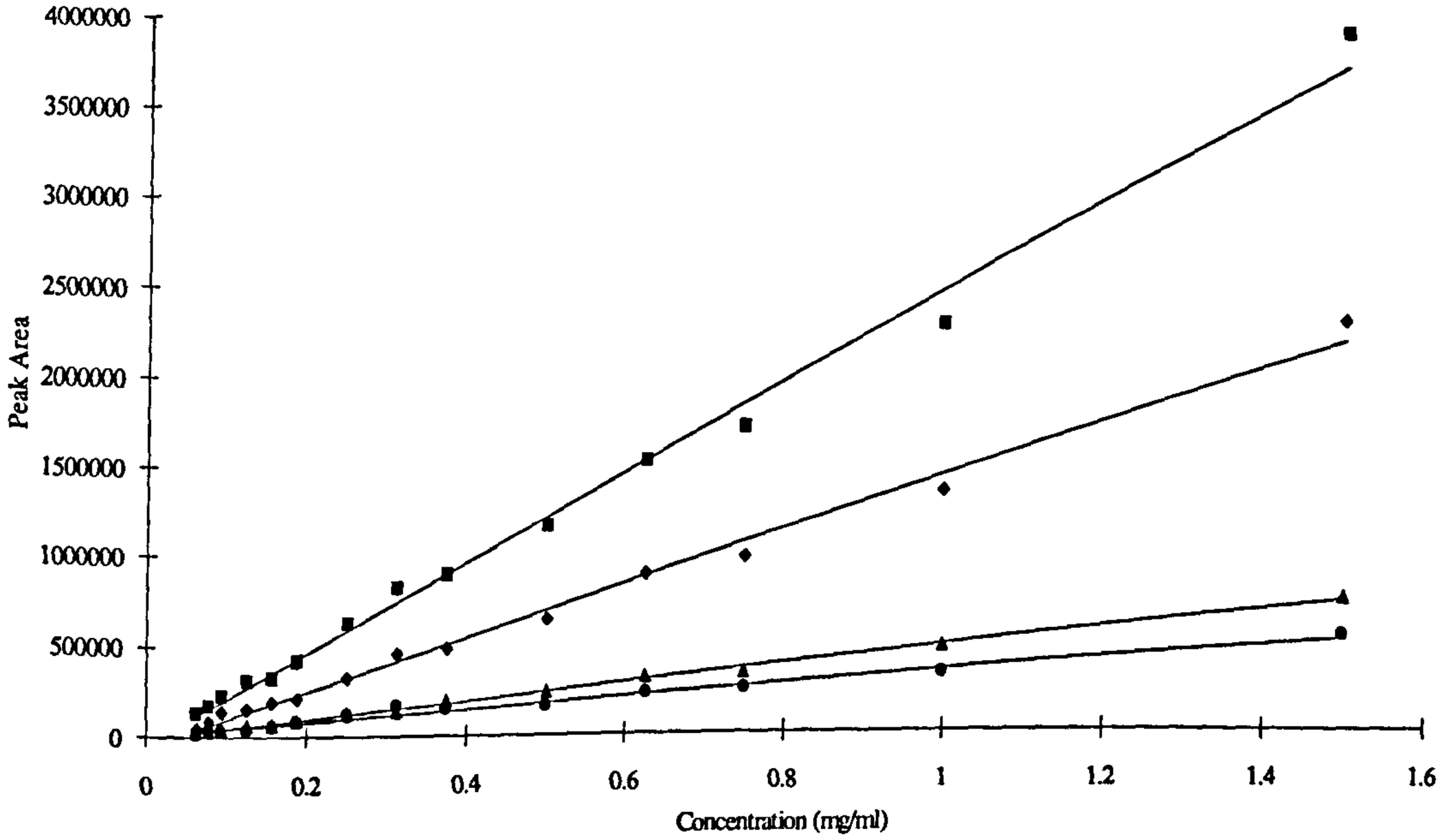
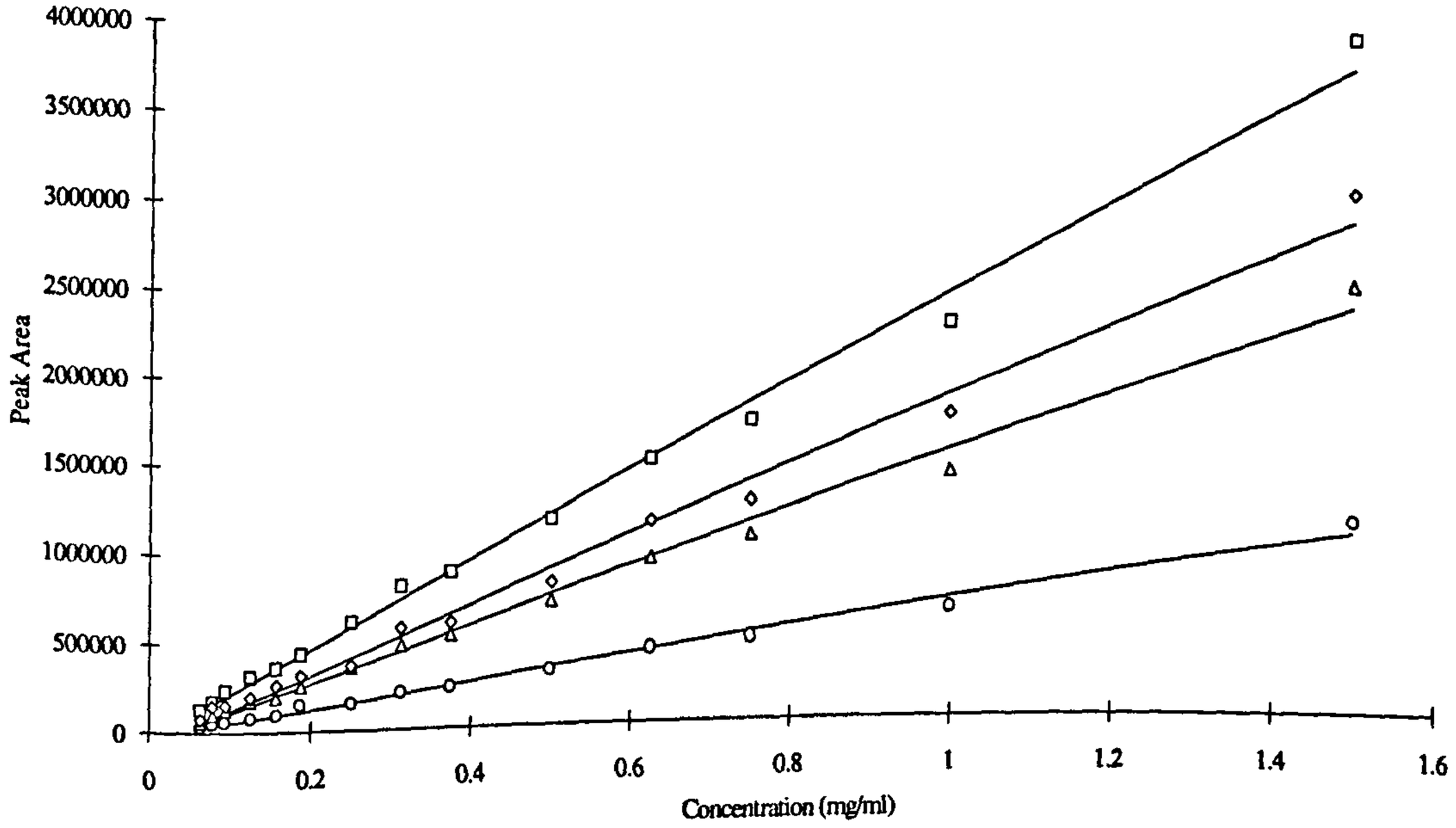


Figure 2.1.6.3.1 d



2.1.6.3.2 High Perfomance Chromatographic Analysis Of Four Commercially Available "Pure" Sulphides; DADS, DAS, DMT & DMD

Four commercially available "pure" sulphides; DADS, DAS, DMT and DMD were obtained for assessment of their antimicrobial activity. Commercial literature claimed a high degree of purity (>90%). Subsequent HPLC analyses of each of these sulphides (Figures 2.1.6.3.2a, b, c, d) revealed them to contain significant levels of impurity.

Estimated purity assessments of these commercial sulphides by HPLC according to 2.1.6.3 (based upon GC analysis results correlated with HPLC peak areas for G.O) are given in Table 2.1.6.3.2e. A tentative identification of contaminating components based upon comparison with respect to retention times of known G.O components was made.

Table 2.1.6.3.2e Purity Assessments Of Commercial Sulphide Preparations

	Commercial	Sulphide	Preparations	
Percentage Component/ Contamination	Diallyl sulphide (DAS)	Diallyl disulphide (DADS)	Dimethyl disulphide (DMD)	Dimethyl trisulphide (DMT)
Percentage Component	83%	33%	99%	approx 100%
Percentage Contamination				
Diallyl sulphide	- -	33%	0	0
Diallyl disulphide	7.8%	- -	0.68%	0
Diallyl trisulphide	3.0%	16.5%	0.32%	0
Diallyl tetrasulphide	6.2%	17.5%	0	0

Figure 2.1.6.3.2a: HPLC Analysis Of Diallyl disulphide (DADS)

Figure 2.1.6.3.2b: HPLC Analysis Of Diallyl sulphide (DAS)

Figure 2.1.6.3.2c: HPLC Analysis Of Dimethyl disulphide (DMD)

Figure 2.1.6.3.2d: HPLC Analysis Of Dimethyl trisulphide (DMT)

10mg of the commercial sulphide preparations were extracted with separate 10ml of ACN, the extracts were then filtered through an HPLC filter and 10µl of the individual extracts injected directly into the HPLC.

Conditions Of HPLC:

1) ODS2 Column

2) Mobile Phase = ACN:H_2O:THF (70:27:3)

3) Flow Rate = 1.0ml/min

4) UV detector absorbance at a wavelength of 254nm

Figure 2.1.6.3.2a

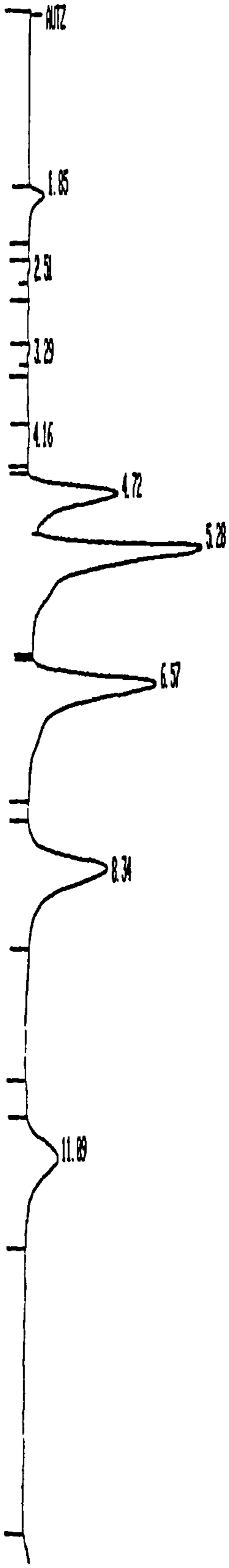


Figure 2.1.6.3.2b

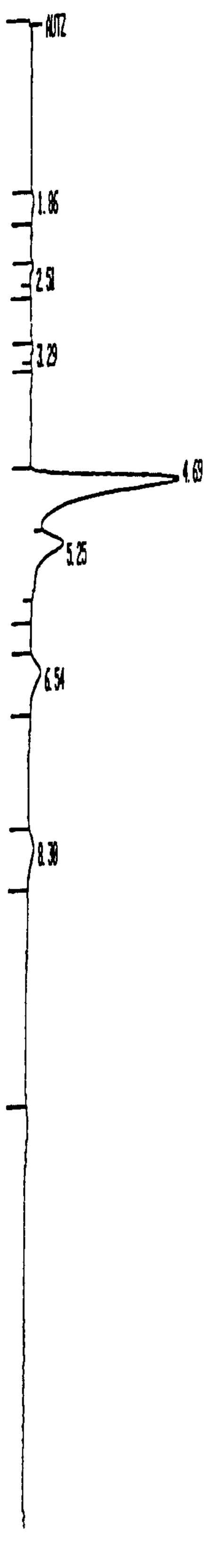


Figure 2.1.6.3.2c

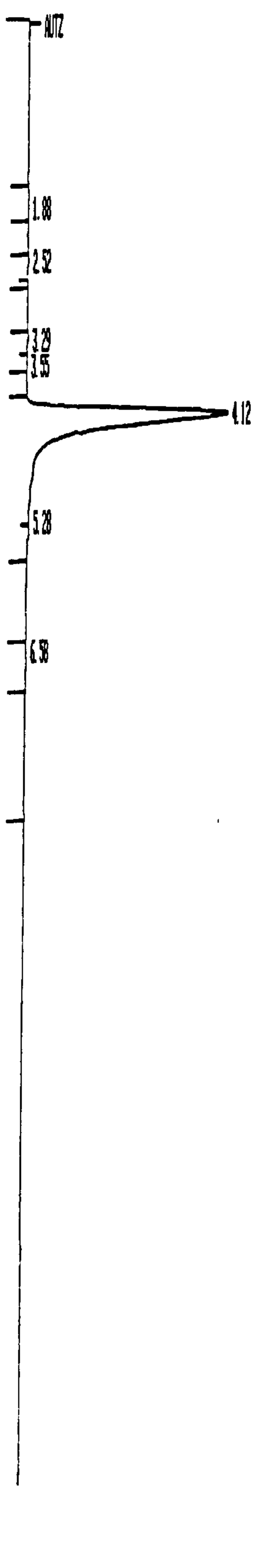
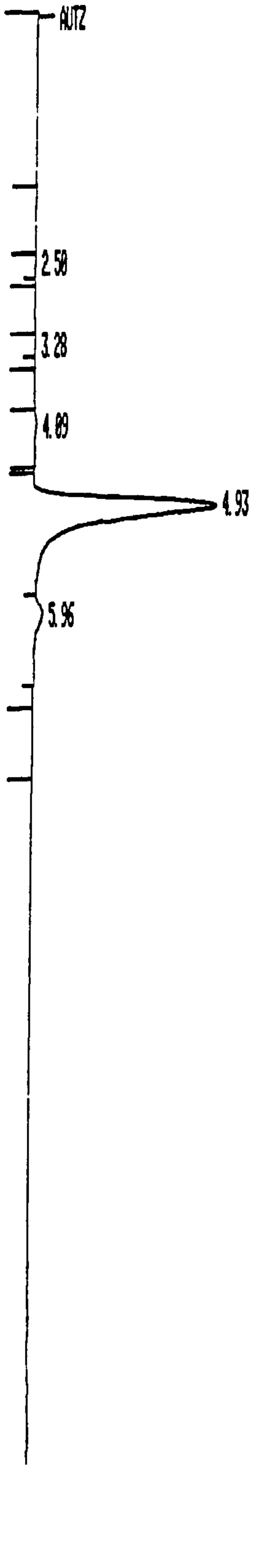


Figure 2.1.6.3.2d



2.1.6.4 High Performance Liquid Chromatographic Analysis Of Allicin From Freeze-Dried Garlic Powder

1g of freeze-dried G.P was homogenised with 30ml of water using an Ystral homogeniser for 1 minute on ice. The homogenate was then stood for 30 mins at room temperature to allow alliin conversion to allicin. This was then centrifuged at 10,000 rpm (MSE Europa M24) for 15 mins at 4°C. The supernatant collected was then filtered prior to injection.

2.1.6.4.1 HPLC Conditions

Analyses were performed using a Gilson HPLC system with PYE UNICAM PU4020 UV Detector (Phillips) and Trio Integrator (at Humberside University) and by a LKB Bromma HPLC System with a 2152 Controller, 2150 HPLC Pump, 2158 UVICORD SD and 2220 Recording Integrator (at Wolverhampton University). A Spherisorb ODS-2 column with a flow rate of 1ml/min, MethOH:H_2O, (METHANOL:WATER) (50:50) was used as the mobile phase, with the UV detector set at 254nm.

2.1.7 Determination Of The Effect Of Garlic Products On Enzymatic Activity

The effects of both G.O and G.P (and two sulphide components of the oil; DADS and DMD) on the activities of the enzymes; Lactate dehydrogenase and Alcohol dehydrogenase were measured.

Serial dilutions of the garlic products were prepared in double distilled water. 200µl of the various product concentrations were incubated at 25°C with 200µl of the assay buffer containing the enzymes, the activity of which was then measured at regular time intervals.

2.1.7.1 Lactate Dehydrogenase (LDH)

LDH was obtained from Sigma as a commercial preparation from Rabbit muscle.

$$\text{D-Lactate} + NAD^+ \leftrightarrow \text{Pyruvate} + NADH + H^+$$

LDH activity was determined by monitoring the oxidation of NADH to NAD in the presence of pyruvate as substrate at 25°C.

The assay mixture was as follows:

Reagent	Concentration in assay
3.00 ml Phosphate buffer (0.1 M; pH 7.0)	94.5 mM
0.10 ml Pyruvate, Na salt (2.5 mg/ml)	0.77 mM
0.05 ml NADH, Na salt (10 mg/ml)	0.2 mM
0.02 ml Enzyme solution in buffer	100 U/ml

NADH oxidation was monitored at a wavelength of 340nm (ε_{NADH}= 6.22x10^3) using a PYE UNICAM SP 1800 Spectrophotometer linked to a PYE UNICAM Detector and a 3.17ml cuvette with a path length of 1cm. The change in absorbance was read against a blank containing distilled water instead of the substrate.

2.1.7.2 Alcohol Dehydrogenase (ADH)

ADH was obtained from Sigma, as a commercial preparation from yeast.

$$C_2H_5OH + NAD^+ \leftrightarrow CH_3CHO + NADH + H^+$$

ADH activity was determined by monitoring the reduction of NAD to NADH in the presence of ethanol as the substrate at 25°C.

The assay mixture was as follows:

Reagent	Concentration in assay
2.50 ml Sodium pyrophosphate buffer	85.5 mM
(0.1 M; pH 9.0; containing	
1.67 mg. glycine/ml)	19.1 mM glycine
0.10 ml Semicarbazide hydrochloride	6.2 mM
(250 mg./ml; pH 6.5)	
0.10 ml Ethanol (96%)	0.6 mM
0.20 ml NAD (20 mg./ml)	1.8 mM
0.01 ml GSH (90 mg./ml)	1.0 mM
0.02 ml Enzyme solution in 0.1% albumin solution	100 U/ml

NAD reduction was monitored at a wavelength of 340nm (ε_{NAD}= 6.22x10^3) using a PYE UNICAM SP 1800 Spectrophotometer linked to a PYE UNICAM Detector and a 2.93ml cuvette with a path length of 1cm. The change in absorbance was read against a blank containing distilled water instead of the substrate.

2.2 MATERIALS

2.2.1 Commercial Garlic Products

1. Steam distilled garlic oil (G.O), obtained from Seven Seas Ltd, Marfleet, Hull, West Yorkshire.

2. Freeze-dried garlic powder (G.P), obtained from Interprise Ltd, Port Talbot, West Glamorgan.

2.2.2 Microorganisms

All bacterial cultures were obtained from the University of Wolverhampton culture collection.

2.2.3 Chemicals

Analar grade chemicals from BDH and Sigma were used wherever possible. Other chemicals were obtained from the following manufacturers as follows:-

Aldrich Chemical Company Ltd., Gillingham, England.

API-BioMérieux SA, Lyon, France.

BDH Ltd., Atherstone, Warwickshire, England.

Boehringer Mannheim UK., Lewes, East Sussex, England. (LDH)

Difco Laboratories Ltd., East Molesey, Surrey, England. (BHI, CA, LSA, TJA, TSA)

Fluka Chemicals, Gillingham, Dorset, England. (DAS and DADS)

Gibco Ltd., Paisley, Scotland. (BA)

Lab M, (Amersham International UK.), Bury, Lancashire, England. (MC and MCNS)

Oxoid Ltd., Basingstoke, Hampshire, England. (TSB)

Pfaltz & Bauer, (Phase Separations Ltd.), Deeside, England. (DMD and DMT)

Sigma Chemical Company Ltd., Poole, England. (LDH and ADH)

CHAPTER 3

RESULTS

3.0 RESULTS

SECTION 1 - Antimicrobial Activity Of The Garlic Products

It is important to note that any method of determining the antimicrobial activity of the garlic products within a liquid growth medium should facilitate the direct contact of the garlic products with the microorganism. Initial experiments (Maslin & Hill, 1990) showed that garlic oil (G.O) was immiscible with TSB and tended to settle out of solution during incubation of the tubes. This might prevent the complete antimicrobial activity of the garlic products from being exerted. Therefore methods of incorporating G.O as a fine emulsion into the test medium were investigated by; 1) application of detergents AND 2) agitation of the test medium. It was concluded that in practice neither of these methods were improvements upon the simple addition of garlic products directly to the medium.

3.1 Minimal Inhibitory Concentration Determinations (MIC)

Initial triplicate MIC determinations were performed in order to provide a relatively quick means of assessing the susceptibility of a wide range of microorganisms towards antimicrobial properties of the garlic products.

A variety of microorganisms (non-pathogenic and pathogenic, of which those identified as enteric isolates are denoted by *) have been screened in this study for their susceptibility to either G.O or garlic powder (G.P) and a range of MIC values obtained, as shown in Tables 3.1a-d. All values quoted are from MIC tests performed on at least two separate occasions. As can be seen MIC values obtained with both G.O and G.P were highly reproducible. Considering all organisms, a range of MIC values from 5.5 down to 0.01mg/ml was obtained with G.O after 24 hours incubation and of 5.5 down to 0.02mg/ml after 48 hours, while with G.P a range of 25 down to 3.125mg/ml was obtained in the cases of 24 and 48 hours incubation.

Table 3.1a MIC Determinations Of G.O Against Gram +ve Bacteria

* denotes enteric isolates

	MIC VALUE mg/ml	
Organism (University Culture Collection No.)	After 24 hours	After 48 hours
Gram +ve organisms		
Bacillus sp (17)	0.68	0.68
Bacillus cereus (10)	0.08	0.17
B. subtilus (20)	0.17	0.68
B. subtilus (21)	0.01	0.17
B. subtilus (22)	0.17	0.17
B. subtilus (52)	0.17	0.17
Lactobacillus acidophilus (L6)	0.17	0.34
L. acidophilus (L8)	1.37	1.37
L. acidophilus (L9)	2.75	2.75
L. acidophilus (456)	2.75	2.75
L. acidophilus (Gillilands)	2.75	2.75
**Listeria monocytogenes (433a)*	0.02	0.08
**L. monocytogenes (433b)*	0.02	0.02
Staphylococcus aureus (77)	1.37	1.37
Staph. aureus (96)	2.75	5.5
Staph. aureus (298)	2.75	5.5
Staph. aureus (332)	0.34	0.34
Staph. aureus (360)	1.37	1.37
Streptococcus sp (202)	0.08	0.17
**Streptococcus faecalis (477a)*	0.34	0.34
**Strep. faecalis (477b)*	0.34	0.34
Strep. mutans (273)	0.08	0.08
Strep. pyrogenes (94)	0.02	0.04

Table 3.1b MIC Determinations Of G.O Against Gram -ve Bacteria

* denotes enteric isolates

	MIC VALUE mg/ml	
Organism (University Culture Collection No.)	After 24 hours	After 48 hours
Gram -ve organisms		
Bacteroides fragilis (333a)	0.04	0.04
Bacteroides fragilis (333b)	0.02	0.04
Enterobacter aerogenes (3)	0.68	0.68
**Enterococcus faecalis (478)*	0.34	0.34
Escherichia coli st B (45)	0.68	5.5
E. coli EMG 27 (44)	0.34	0.68
**E. coli 055 (427)*	5.5	5.5
**E. coli 0128 (428)*	2.75	2.75
**E. coli 112AC (429)*	2.75	5.5
E. coli (36)	1.37	2.75
E. coli (37)	0.34	0.34
E. coli (38)	0.34	0.34
E. coli (40)	0.68	2.75
E. coli (45)	0.34	0.34
E. coli (97)	2.75	5.5
E. coli (218)	2.75	5.5
E. coli (219)	2.75	5.5
E. coli (222)	2.75	5.5
E. coli (223)	2.75	5.5
E. coli (224)	0.68	0.68
E. coli (225)	0.34	0.68
E. coli var communior (46)	0.68	2.75
Klebsiella aerogenes (325)	0.04	0.17
Proteus vulgaris (201)	1.37	2.74

Table 3.1b Continued

* denotes enteric isolates

	MIC VALUE mg/ml	
Organism (University Culture Collection No.)	After 24 hours	After 48 hours
Gram -ve organisms continued		
**Salmonella enteritidis (416a)*	0.34	0.34
**S. enteritidis (416b)*	5.5	5.5
**S. enteritidis 09 gm pt4 (417)*	5.5	5.5
**S. infantis 06, 7r1,5 (418)*	2.75	5.5
**S. senftenberg (419a)*	5.5	5.5
**S. senftenberg (419b)*	5.5	5.5
**S. typhimurium (434a)*	0.34	0.68
**S. typhimurium (434b)*	2.75	5.5
**S. typhimurium 04i 1,2 (435)*	1.37	2.75
**Shigella boydii (421)*	1.37	2.75
**Shigella flexnelri (422)*	1.37	2.75
**Shigella sonnei (221)*	2.75	2.75
**Shig. sonnei (424)*	2.75	5.5
**Shig. sonnei (423)*	2.75	2.75
**Shig. sonnei (426)*	2.75	2.75
Vibrio sp (Non Cholera) (439)	0.34	0.68
**V. fluvialis (436)*	2.75	2.75
**V. metshnikon (437)*	0.02	0.34
**V. parahaemolytica (438)*	0.04	0.08
**Yersinia enterocolytica ( )*	0.01	0.02
**Y. enterocolytica (431)*	0.34	0.68
**Y. enterocolytica (432)*	0.17	0.68

Table 3.1c MIC Determination Of G.P Against Gram +ve Bacteria

* denotes enteric isolates

	MIC VALUE (mg/ml)	
Organism (University Culture Collection No.)	After 24 hours	After 48 hours
Gram +ve organisms		
Bacillus sp. (17)	6.25	12.5
Lactobacillus acidophilus (L6)	6.25	12.5
L. acidophillus (L8)	6.25	12.5
**Listeria monocytogenes (433a)*	6.25	25
**L. monocytogenes (433b)*	6.25	25

Table 3.1d MIC Determination Of G.P Against Gram -ve Bacteria

* denotes enteric isolates

	MIC VALUE (mg/ml)	
Organism (University Culture Collection No.)	After 24 hours	After 48 hours
Gram -ve organisms		
Bacteroides fragilis (333)	6.25	6.25
Enterobacter aerogenes (3)	6.25	6.25
**E. coli 055 (427)*	12.5	25
**E. coli 0128 (428)*	12.5	25
**E. coli 112AC (429)*	3.125	3.125
E. coli (40)	6.25	12.5
E. coli st B (45)	3.125	25
E. coli var communior (46)	6.25	6.25
E. coli NCIB 8112 (47)	12.5	12.5
Klebsiella aerogenes (48)	12.5	12.5
**Salmonella enteritidis (416a)*	6.25	12.5
**S. enteritidis (416b)*	6.25	12.5
**S. enteritidis 09 gm pt4 (417)*	6.25	12.5
**S. infantis 06,7r1,5 (418)*	6.25	25
**S. senftenberg (419a)*	12.5	25
**S. typhimurium (434a)*	6.25	12.5
**S. typhimurium (434b)*	6.25	12.5
**S. typhimurium 04i 1,2 (435)*	12.5	25
Serratia marcesans (75)	3.125	6.25
**Shigella boydii (421)*	6.25	6.25
**Shig. flexnelri (422)*	6.25	6.25
**Shig. sonnei (424)*	12.5	12.5
**Shig. sonnei (423)*	12.5	12.5
**Shig. sonnei (426)*	12.5	12.5
**Yersinia enterocolytica (431)*	6.25	6.25
**Y. enterocolytica (432)*	6.25	6.25

Closer inspection of the G.O MIC results reveal that variation in MIC values occur within strain isolates of a species, especially *E. coli*. From Table 3.1e, it can be seen that the MIC values obtained for the strains of certain species fall within certain ranges; *E. coli* (0.34-5.5mg/ml), *Staph. aureus* strains (0.34-2.75mg/ml) which contrast to those of the more susceptible *Streptococcus spp* (0.02-0.34mg/ml). Note also that comparison of the MIC values between non- and pathogenic strains of *E. coli* species does not indicate significant differences.

Table 3.1e G.O MIC Ranges With Respect To Species

Bacterial Species (No. of Isolates tested)	MIC Range mg/ml	Mean mg/ml
Bacillus spp (6)	0.01 - 0.68	0.21
Bacteroides fragilis (2)	0.02 - 0.04	0.03
**Escherichia coli (5)*	0.34 - 5.5	1.87
Escherichia coli (13)	0.34 - 2.75	1.37
Lactobacillus acidophilus (5)	0.17 - 2.75	1.95
**Listeria monocytogenes (2)*	0.02 - 0.02	0.02
**Salmonella typhimurium (3)*	0.34 - 2.75	1.48
**Salmonella enteritidis (3)*	0.34 - 5.5	3.78
**Shigella spp (6)*	1.37 - 2.75	2.29
Staphylococcus aureus (5)	0.34 - 2.75	1.71
Streptococcus spp (5)	0.02 - 0.34	0.03
Vibrio spp (3)	0.02 - 2.75	0.93
**Yersinia enterocolytica (3)*	0.01 - 0.34	0.17

It can be seen that certain pathogenic bacteria such as *L. monocytogenes* and *Y. enterocolytica* appear to be very sensitive to G.O while *E. coli*, *E. aerogenes* and *L. acidophilus*, which are bacteria that naturally inhabit the intestinal tract (playing an important role in the maintenance of a healthy digestive system) appear to be less sensitive to G.O. It was also noted that the species of *Salmonella* tested (which are known causative agents of food poisoning) appear to be less sensitive.

The G.P MIC results (Table 3.1f) show more discrete ranges than those of G.O for the same organisms (Table 3.1e) indicating more limited variation in sensitivity, than is the case for G.O.

Table 3.1f G.P MIC Ranges With Respect To Species

Bacterial Species (No. of Isolates tested)	MIC Range mg/ml	Mean mg/ml
**Escherichia coli (3)*	3.125 - 12.5	8.6
Escherichia coli (4)	3.125 - 12.5	7.2
L. acidophilus (3)	6.25 - 6.25	6.25
**Listeria monocytogenes (2)*	6.25 - 6.25	6.25
**Salmonella typhimurium (3)*	6.25 - 12.5	8.3
**Salmonella enteritidis (3)*	6.25 - 6.25	6.25
**Shigella spp (5)*	6.25 - 12.5	10
**Yersinia enterocolytica (2)*	6.25 - 6.25	6.25

3.1.1 Comparison Of The Potencies Of G.O & G.P

(For example calculations See Appendix 2).

Data utilized in potency comparisons - 24 hour MIC values.

Strains of bacteria used; *E.coli* (40), *S. typhimurium* (435), *L. monocytogenes* (433) and *B. fragilis* (333).

MIC g/L Broth:	*E. coli*	*S. typhimurium*	*L. monocytogenes*	*B. fragilis*
G.O	0.68	1.37	0.02	0.04
G.P	6.25	6.25	6.25	6.25

Relative Potency Values:

A. Weight of Product required for MIC

	E. coli	*S. typhimurium*	*L. monocytogenes*	*B. fragilis*
G.P:G.O	9.19	4.56	312.5	156.25

B. Weight of Garlic Bulbs required for MIC (g. Garlic/ml Broth)

	E. coli	*S. typhimurium*	*L. monocytogenes*	*B. fragilis*
G.O	1.08	2.19	0.032	0.064
G.P	0.015	0.015	0.015	0.015

C. Weight of Recommended Dose required for MIC

	E. coli	*S. typhimurium*	*L. monocytogenes*	*B. fragilis*
G.P/G.O	43.52	87.72	1.28	2.56

Comparison of the two garlic products analysed on a "weight of product" basis, indicated that G.O is more potent than G.P, especially for the organisms *L. monocytogenes* and *B. fragilis*, whereas on a "weight of garlic bulbs required for MIC" basis, G.P was more potent than G.O. However, when relative potency according to "weight of product" and recommended dose is calculated, a factor which reflects the relative potency per recommended dose is derived, shows considerably higher values for G.P than G.O, although these vary markedly with species of microorganism tested.

The potency comparisons are based upon data obtained from the MIC determinations and it should be noted that even though the same MIC methodology was used in both cases, such comparisons may not be entirely valid as differences are apparent between G.P and G.O with respect to their properties within aqueous systems (as stated previously p70).

3.1.2 The Effect Of G.O Solubility On Expressed Antimicrobial Activity

As stated on p70, initial observations indicated that G.O was immiscible with TSB and settled out of solution, suggesting that direct contact of the microorganisms with G.O may not occur. Results obtained from MIC determinations with *E. aerogenes* performed on 3 fractions (top, middle and bottom) of a 10% G.O suspension in TSB indicated an identical MIC value of the bottom fraction of 0.68mg/ml to that of "fresh" G.O. Moreover no end point MIC value was obtained with the top and middle fractions (>5.5mg/ml) suggesting that a continual diffusion of the antimicrobial components of G.O occurs from the bottom layer. These results suggest that although G.O is of limited solubility in aqueous media, contact with microorganisms does take place.

3.2 The Effect Of Various Concentrations Of Garlic Products On *E. aerogenes* Cell Viability

Initial analyses of the MIC results after 24 & 48 hours incubation indicated that the effect of garlic products on all the microorganisms tested was not solely inhibition of growth but also involved cell killing (loss of cell viability) particularly at concentrations greater than the MIC value. To further assess the extent of bacteriocidal effects as opposed to bacteriostatic effects, experiments were performed in which the population of viable cells in the presence of various garlic product concentrations was monitored with respect to time. These experiments were performed under identical conditions three times and similar results obtained from each garlic material tested. Typical sets of results are presented in Figures 3.2a, b & 3.2c, d for G.O and G.P respectively.

These viability studies indicated a difference in MIC value obtained to that determined previously (Section 3.1), that is 0.68mg/ml G.O from the MIC method and 1.375mg/ml from the viability study. This difference (a two-fold error, that is a 1 test tube difference) could be a consequence of the differences in experimental technique, such as the repeated disturbance of the medium for sampling purposes in the time dependent studies, or may be within the expected limits of error of these techniques.

The results revealed that the presence of G.O in the growth medium (Figures 3.2a, b) produces distinct phases of response. Concentrations below the MIC value (G.O=0.68mg/ml, G.P=6.25mg/ml) result in a 'lag' phase before *E. aerogenes* reproduce, and a subsequent reduction in the initial growth rate of the cells, as compared to *E. aerogenes* growth in the absence of G.O and G.P. In the presence of G.O concentrations ranging from 1.375 to 0.34mg/ml, an initial lag phase occurs which ceases later and leads to a lower growth rate the higher the G.O concentration. It was noted that the addition of G.O concentrations above 1.375mg/ml and G.P concentrations above 3.125mg/ml, results in cell death (assessed as loss of colony forming units). These results show that both garlic products tested have microbiocidal effects as well as growth inhibiting effects, as was indicated by the MIC results, and that the higher the concentration of garlic product present the greater the initial rate of kill. At the highest concentration of G.O (22mg/ml) neither the 'lag' nor the plateau stages are seen, instead bacterial death commences immediately and the rate of kill declines progressively. In addition there is evidence to suggest that the length of the lag phase and the reduction in the initial growth rate are proportional to the concentration of G.O present.

As with G.O, it was shown that at lethal G.P concentrations (Figures 3.2c, d), the rate and level of cell population death was concentration dependent and at the lethal G.P concentrations the duration of a relative plateau phase present prior to steep population decline was shorter the higher the G.P concentration.

Figure 3.2a: The Effect Of G.O On *E. aerogenes* Viability With Time

Figure 3.2b: Expanded View Over 120 Minutes

Serial two-fold dilutions of G.O were prepared in TSB in tin foil capped boiling tubes, to a total volume of 20ml. 40µl of an overnight culture of *E. aerogenes* was used as an inoculum. The tubes were incubated in a water bath at 37°C and the viability of the cells measured with respect to time using either the spread plate or Miles & Misra enumeration techniques.

LEGEND:

Control (No G.O)
0.34 mg/ml
0.68 mg/ml
1.375 mg/ml
2.75 mg/ml
5.5 mg/ml
11 mg/ml
22 mg/ml

Figure 3.2 a

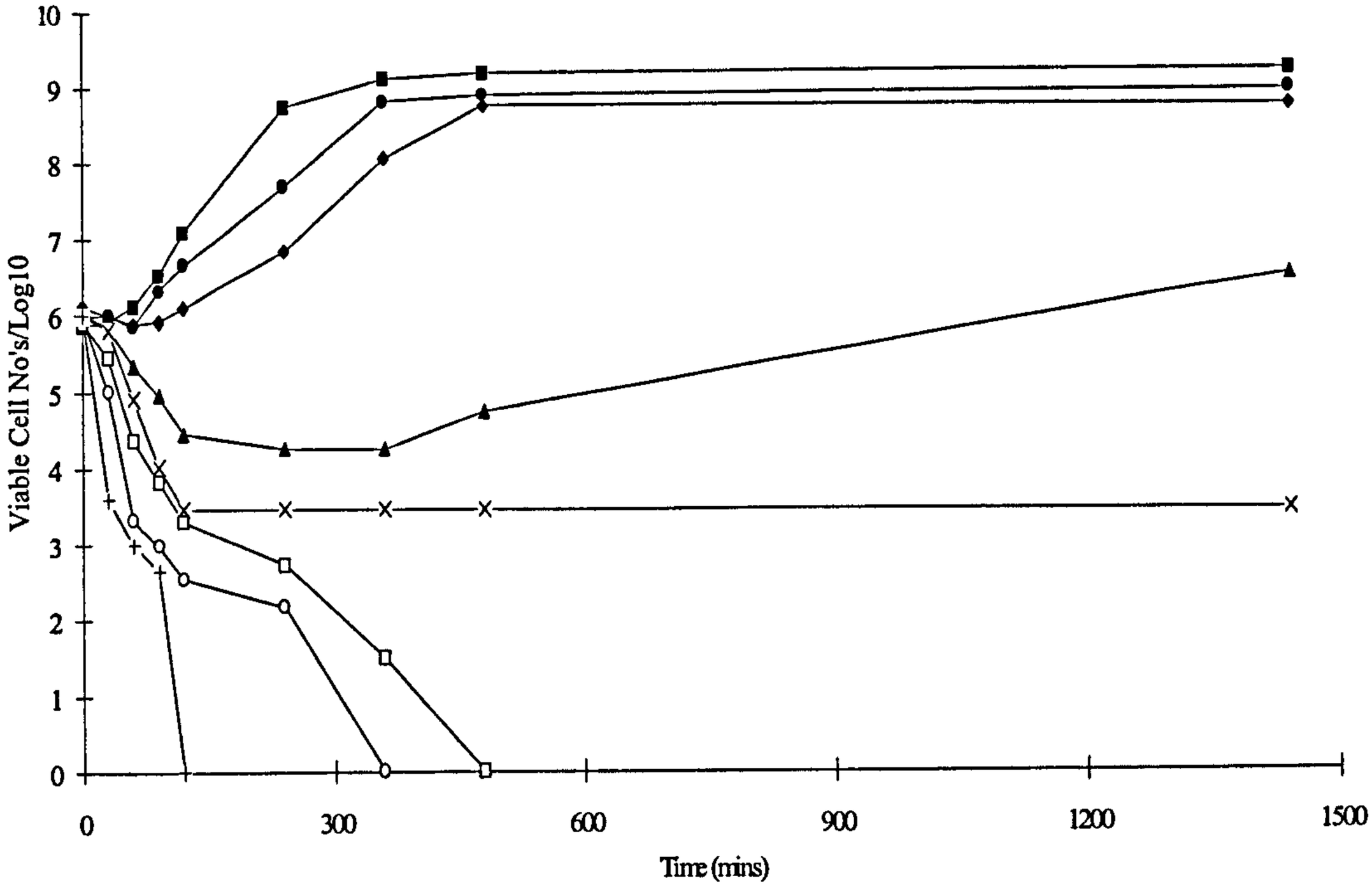


Figure 3.2 b

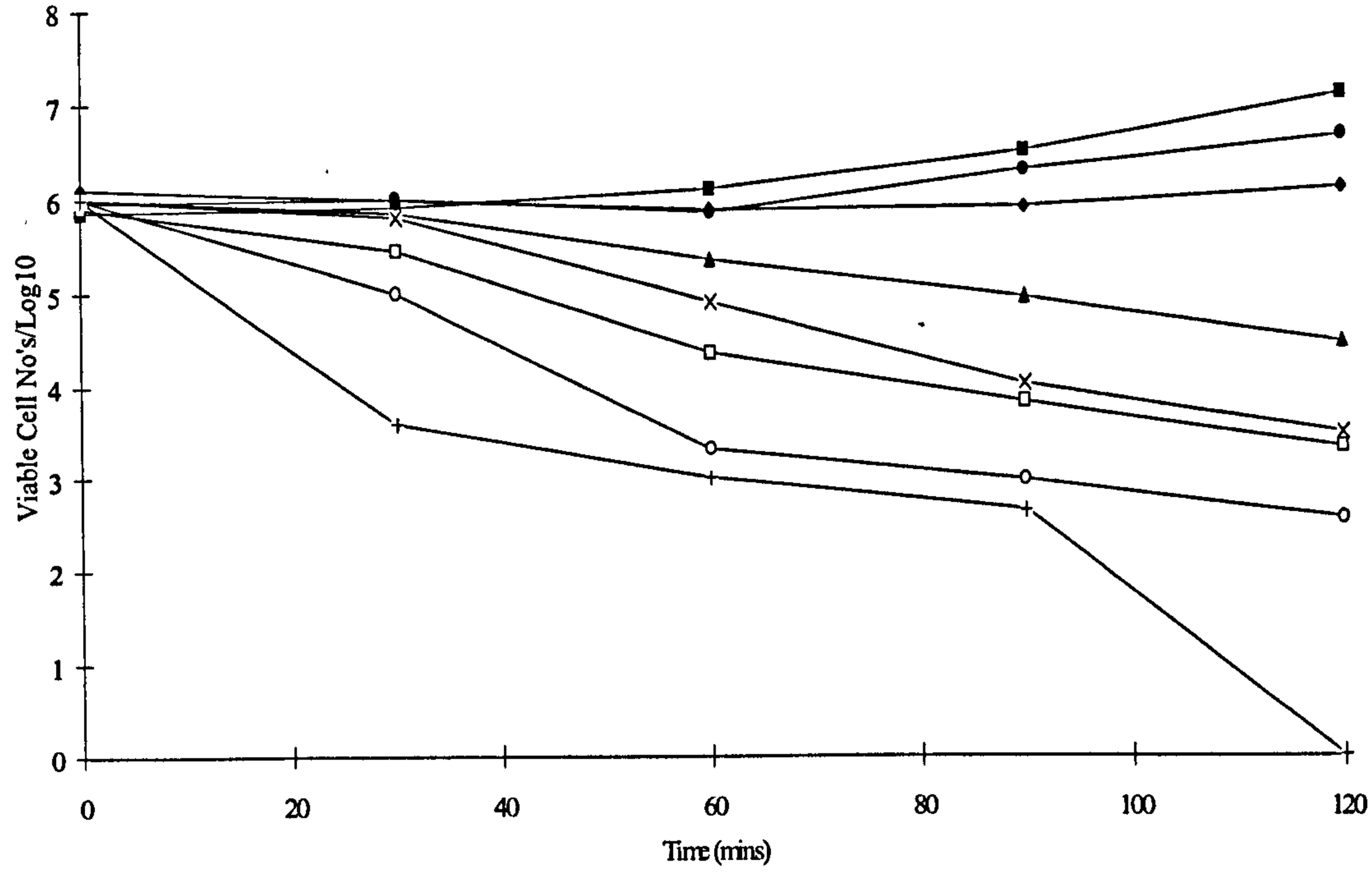


Figure 3.2c: The Effect Of G.P On *E. aerogenes* Viability With Time

Figure 3.2d: Expanded View Over 120 Minutes

Serial two-fold dilutions of G.P were prepared in TSB in tin foil capped boiling tubes, to a total volume of 20ml. 40µl of an overnight culture of *E. aerogenes* was used as an inoculum. The tubes were incubated in a water bath at 37°C and the viability of the cells measured with respect to time using either the spread plate or Miles & Misra enumeration techniques.

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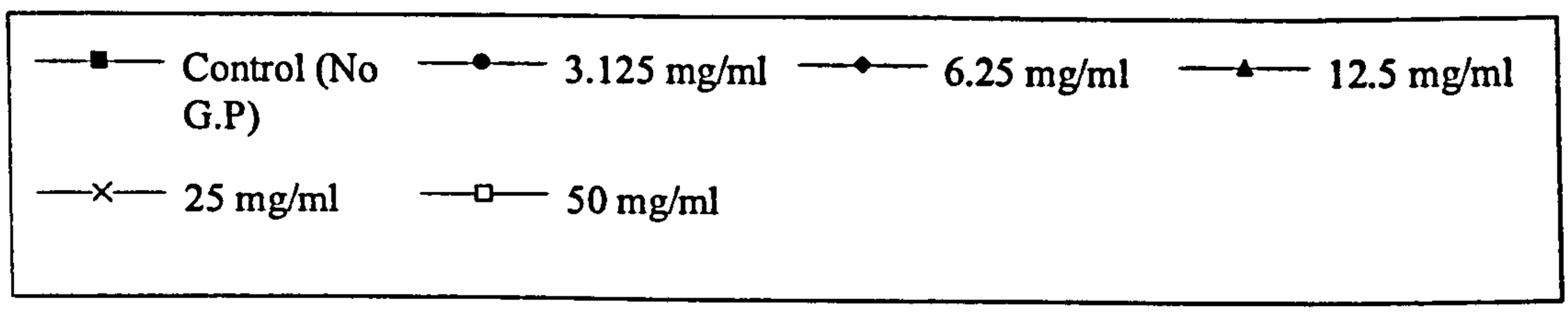


Figure 3.2 c

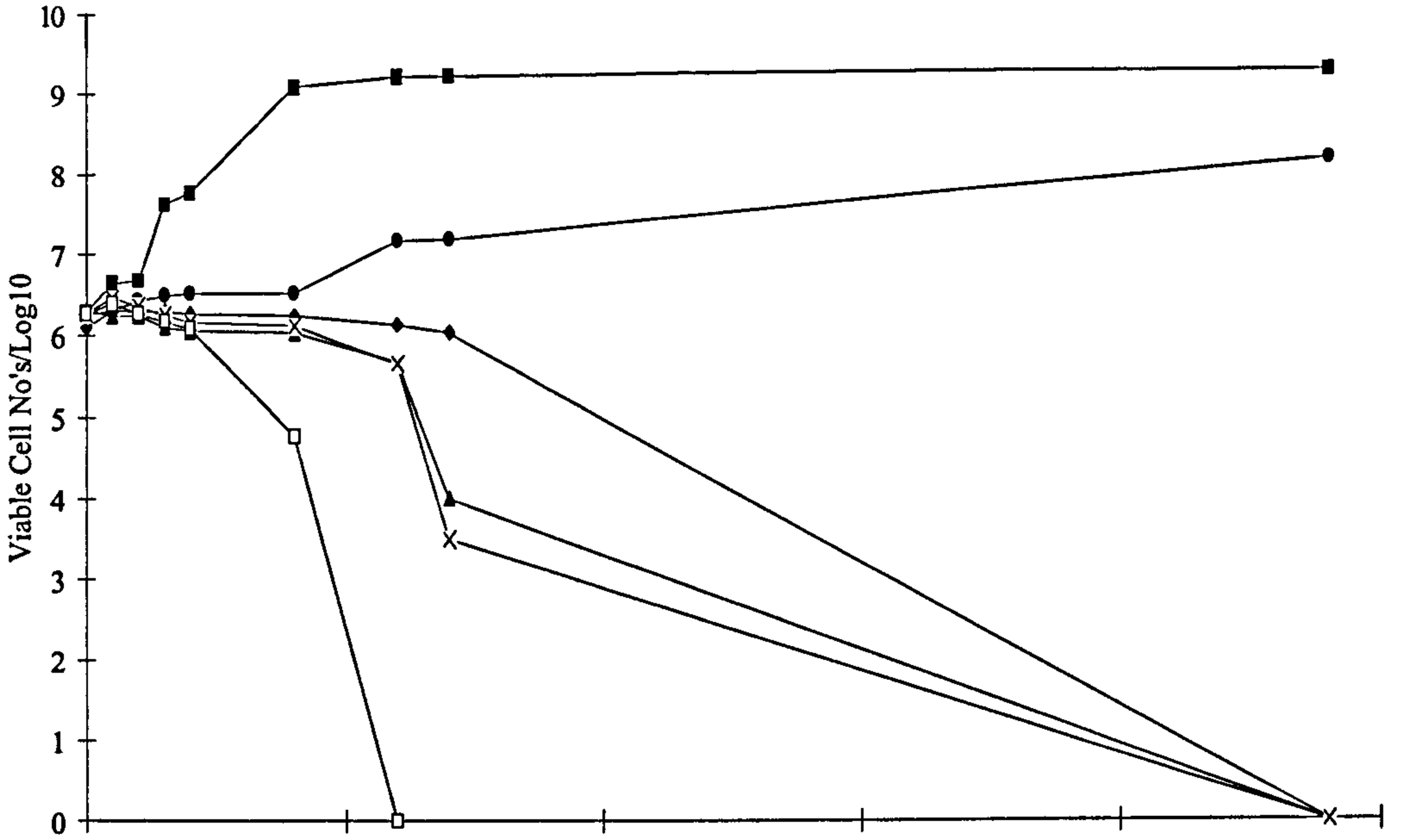
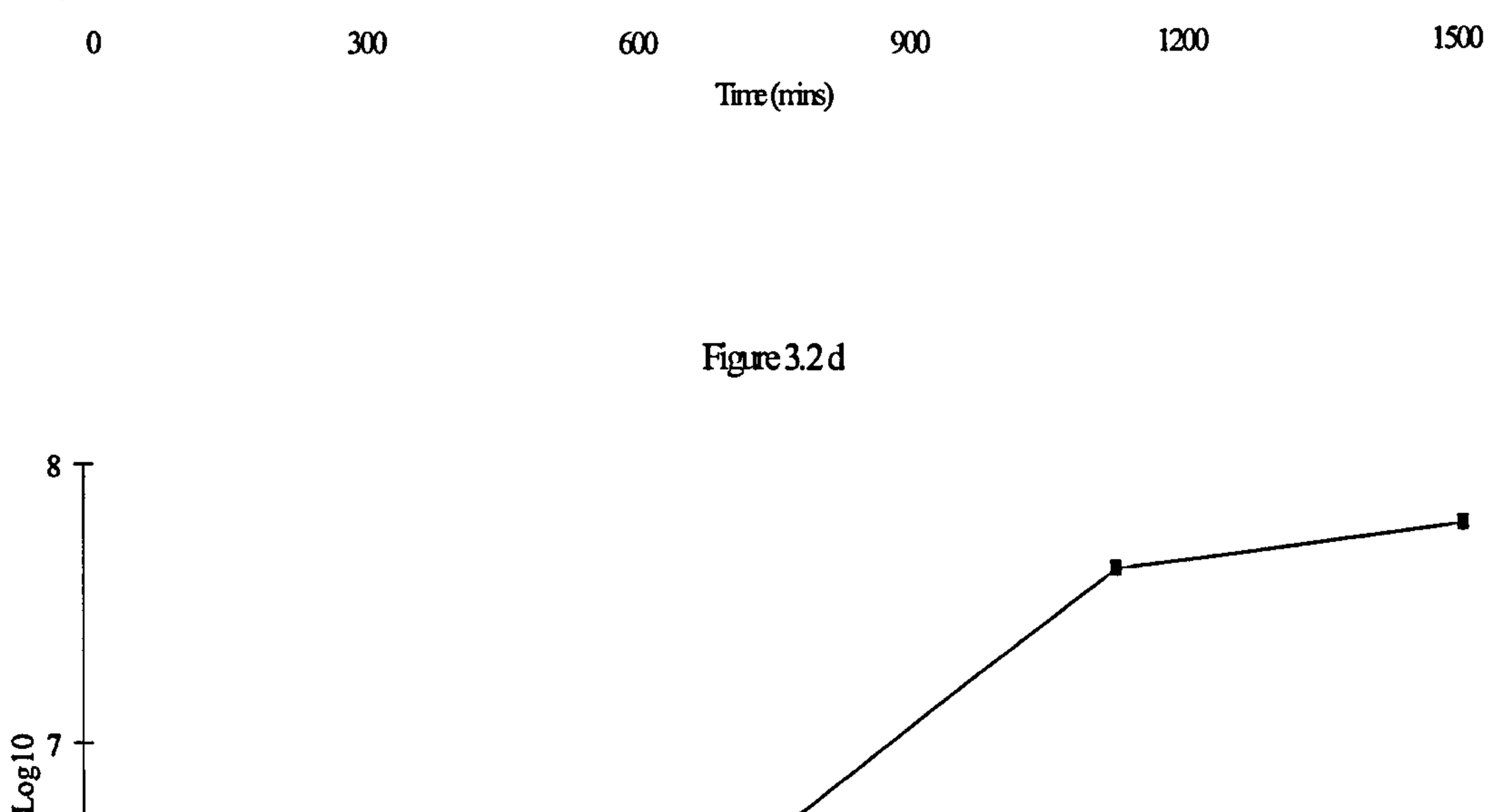


Figure 3.2 d



Utilising the viable count data from the time dependent studies, the relationship between death rate and concentration of garlic product was also examined (See Table 3.2e, Figures 3.2f, g).

The initial cell death rates were calculated, by taking the tangent of the initial slope of the experiment presented in Figure 3.2a, c.

$$\text{CELL DEATH/GROWTH RATE} = \frac{(\text{Log } b - \text{Log } a)}{\text{Log2} \times t}$$

where b = viable cell population at time t_2

a = viable cell population at time t_1

t = time difference (in minutes), $t_2 - t_1$

The -ve values indicate cell death, +ve values indicate cell growth and a 0 value indicates no growth.

Table 3.2e Initial Cell Death/Growth Rates

	Initial Cell Death/Growth Rates mins-1
G.O Concentration (mg/ml)	
22	-0.16
11	-0.12
5.5	-0.10
2.75	-0.09
1.375	-0.04
0.68	-0.044
0.34	+0.02
Control (No G.O)	+0.033
G.P Concentration (mg/ml)	
50	-0.0119
25	-0.00830
6.25	-0.00692
3.125	-0.00138
1.5625	+0.00276
Control (No G.P)	+0.033

Figure 3.2f: The Effect Of G.O On Initial Cell Death/Growth Rates

Figure 3.2g: The Effect Of G.P On Initial Cell Death/Growth Rates

The source of data for these graphs was derived from the initial cell death/growth rates (Table 3.2e) determined from Figures 3.2a & c.

Figure 3.2 f

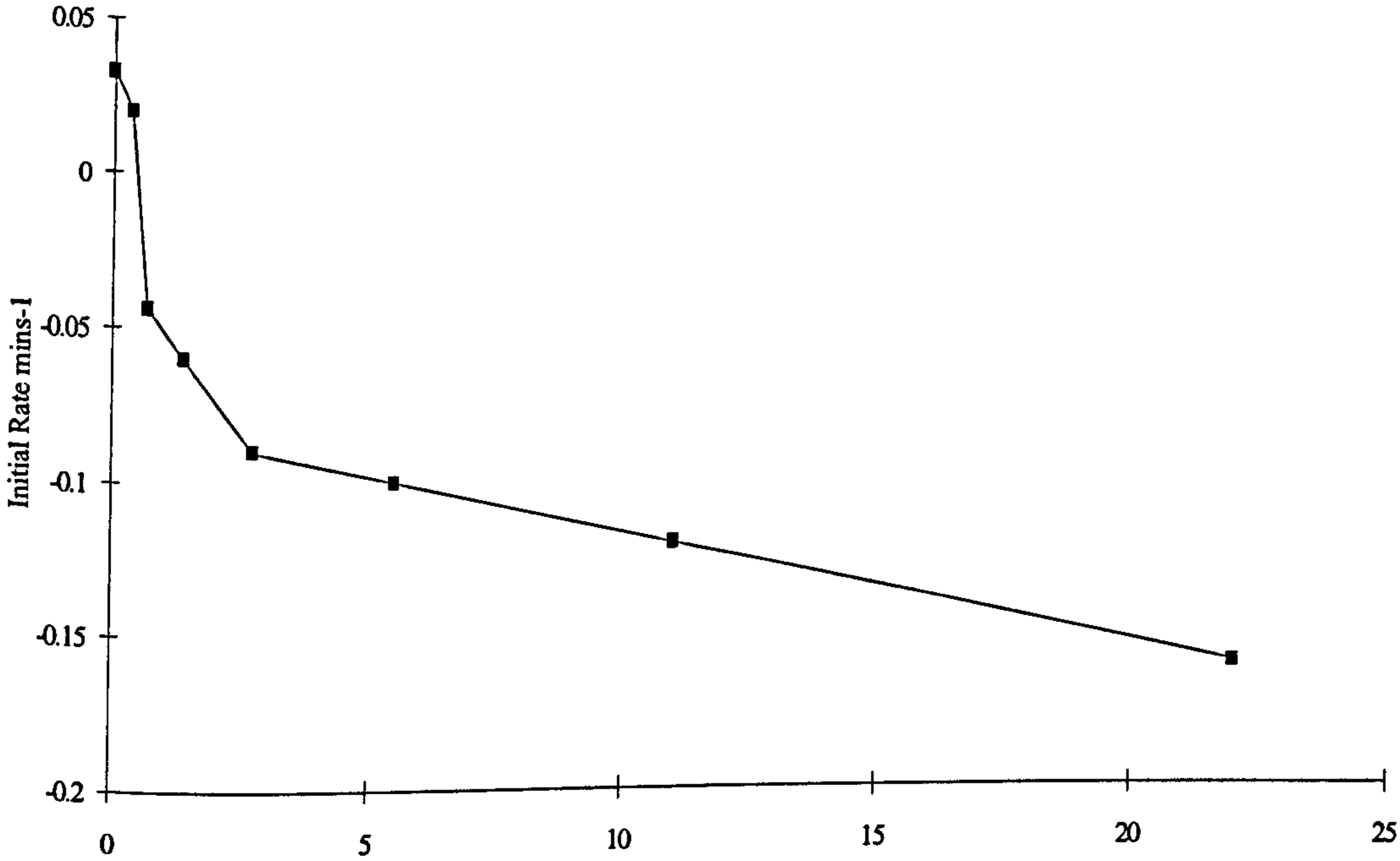
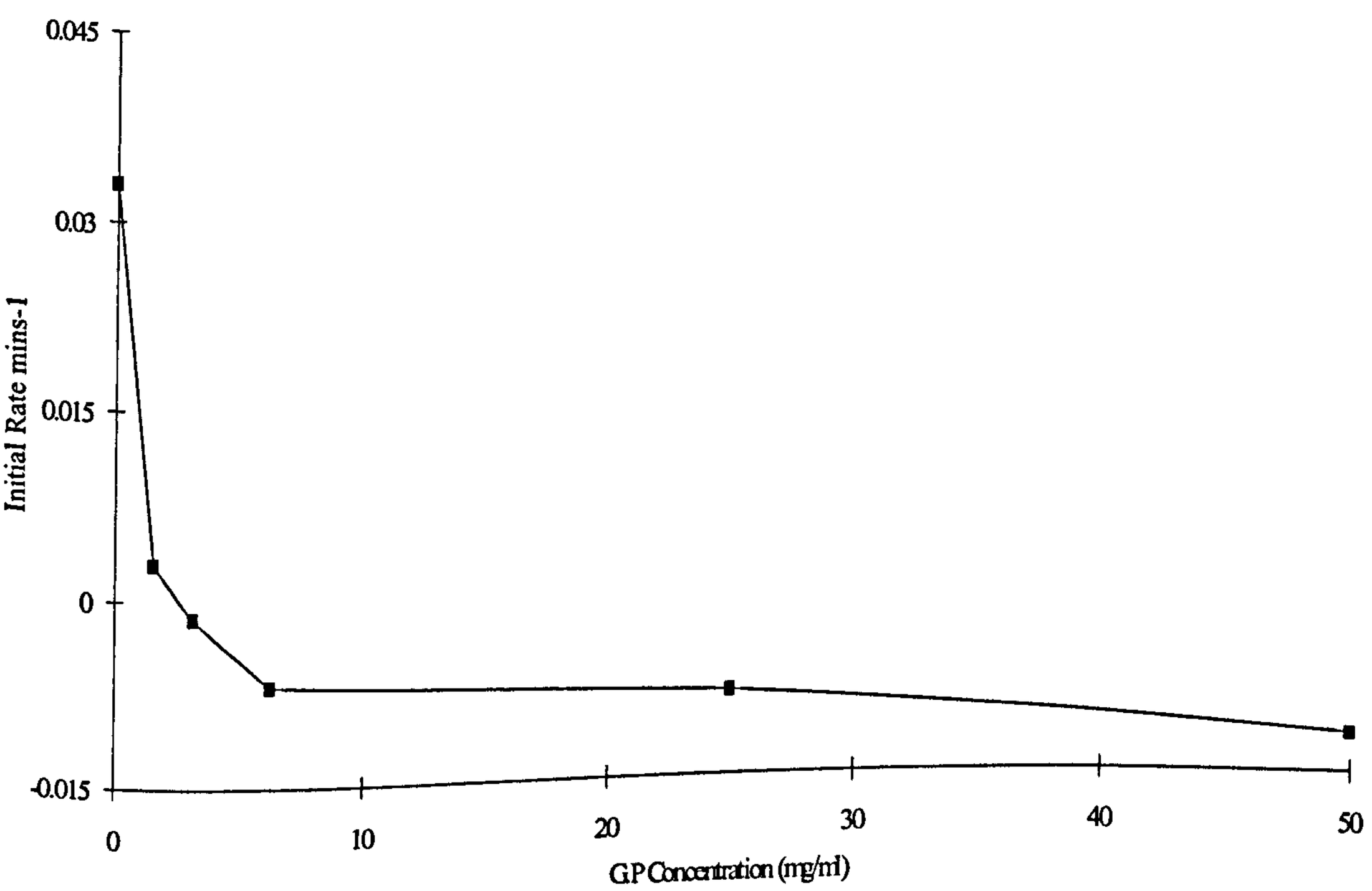


Figure 3.2 g



It can be seen that the initial rate of cell viability loss is proportional to the concentration of garlic products, although this relationship is non-linear. Instead it reveals an inverted hyperbolic relationship between rate of cell population change and concentration of garlic product. Thus the death rates increase progressively more slowly with increasing garlic product concentration. Overall it can be stated that the higher the concentration of G.O or G.P, the greater the proportion of the cell population that is killed, the quicker the population decline (or slower the population growth) and the lower the level at which any population plateau observed stabilises.

The observed growth of cells subsequent to G.O induced growth inhibition or partial cell population death is comparable to the evidence for time dependency found in the MIC studies. This time-dependency has several possible explanations; 1) a time dependent loss, such as volatilisation of the antimicrobial components present in G.O; 2) a progressive inactivation of the antimicrobial compounds resulting from their instability with respect to temperature; 3) an inactivation of the antimicrobial compounds resulting from reaction with chemical components of the surrounding medium AND/ OR 4) the ability of the cells to adapt to the presence of the antimicrobial components in the garlic products.

The following experiments were performed to evaluate the above hypotheses, in addition to providing some evidence for the effect of environmental factors on the antimicrobial activity of G.O; A) the effect of agitation, (See Section 3.3.1); B) the effect of sealed tubes on the antimicrobial activity of the garlic products, (See Section 3.3.2); C) the effect of various growth and non-growth media, (See Section 3.3.3) AND D) the resistance of the microorganisms to the presence of the garlic products, (See Section 3.3.4).

3.3 Determination Of Loss Of G.O Antimicrobial Activity

The aim of this work was to test the hypotheses presented in Section 3.2 (p88).

3.3.1 The Effect Of Agitation (By Forced Aeration/Nitrogenation) On The Antimicrobial Activity Of G.O

These experiments were performed in the belief that agitation could lead to G.O loss by volatilisation (See Hypothesis 1, p88).

Agitation of triplicate 10% G.O suspensions in TSB at 37°C for 4 hours was achieved by forced aeration/nitrogenation at 200cc/min, followed by subsequent triplicate MIC determinations with *E. aerogenes*. Comparisons were made to MIC controls of non-agitated G.O suspensions in TSB. It was observed that an 8-16 fold higher concentration (11-5.5mg/ml) of G.O in the agitated TSB was required to inhibit the growth of *E. aerogenes* than was required with the control (MIC=0.68mg/ml). This suggests that agitation of the G.O (in terms of forced aeration/nitrogenation) suspension leads to loss by volatilisation of a substantial proportion of one or more essential antimicrobial components.

A repeat of the experiment was carried out in sterile double distilled water (SDDW) under the same conditions of agitation, in order to identify what effect, if any, the presence of TSB had upon the effect of agitation upon G.O loss. The difference in the MIC value resulting from aeration and nitrogenation of SDDW compared to fresh G.O was small (1.36mg/ml, a two-fold difference in the MIC value, that is 1 test tube), which is within the expected limits of error for this technique.

These results suggest that the effect of TSB may be to promote dispersion of the G.O in such a way as to promote volatilisation under conditions of agitation. This is feasible as TSB contains substances with hydrophilic/hydrophobic properties such as tryptone, soytone, dextrose and sodium chloride. Alternatively physical or chemical reaction of G.O with TSB components may have removed some of its antimicrobial

activity. An overriding possibility is that conditions for growth and survival of *Enterobacter aerogenes* are so different that the differences in the effect of agitation do not bear significantly upon the possible explanations given in hypotheses 1-3. Further attempts to resolve these alternative explanations are recorded in the following section (3.3.2).

3.3.2 The Effect Of Sealed Tubes On The Antimicrobial Activity Of G.O

The aim of these experiments was to test hypotheses 1 and 2 (p88), by determining the effect of different sealing of the tubes in order to restrict possible volatilisation of the G.O components. The different types of sealing were; 1) foam bung, 2) tin foil, 3) foam bung+tin foil, 4) test tube tops, 5) tight fitting glass stoppers.

Agitation was achieved by prior incubation of triplicate 10% G.O suspensions in TSB at 37°C for 24 hours in a shaking waterbath, while prior static incubation was achieved by placing similar 10% tubes in a 37°C incubator. Triplicate MIC determinations were then performed using the pre-incubated G.O suspensions. Using glass stoppers, both static and agitated prior incubation resulted in the MIC determinations having the same MIC value as determined with fresh G.O (0.68mg/ml). This result indicates that under conditions where G.O could not escape by volatilisation, no loss of G.O detectable by an MIC change was observed. Thus indicates that the hypotheses that G.O loss is due to progressive heat (hypothesis 2) or chemical (hypothesis 3) inactivation may not apply to G.O in TSB, irrespective of whether agitation is provided or not. In contrast it was observed that for both the foam bung and foam bung+tin foil sealed tubes, from either of which G.O might escape, a far higher G.O concentration (11mg/ml) was required to inhibit the growth of *E. aerogenes* after prior agitated incubation compared to the MIC value (0.68mg/ml) after prior static incubation. These results indicate that loss of G.O from TSB by volatilisation (hypothesis 1) is substantial if cap sealing is not gas-tight and agitation is provided. These described results suggest that the change in MIC in TSB reported in the last section (3.3.1) may be explained by loss due to volatilisation alone rather than by physical or chemical combination with medium

components as results obtained in this section (3.3.2) indicate that under static incubation G.O loss was not measurable (in terms of a difference in MIC). It should be noted that similar results were obtained with foil capping only, this is important as it was the technique used in the viability experiments forming much of this study.

What is unresolved by these experiments performed in Section 3.3.1 and 3.3.2 is whether the large increase in MIC due to volatilisation from TSB is enlarged specifically by TSB medium components increasing G.O dispersal. This is especially so in the light of the comparison in Section 3.3.1 of TSB with SDDW, which unlike TSB, is a medium that does not support the growth of *E. aerogenes.*

3.3.3 The Effect Of Chemical Composition Of The Surrounding Media On The Antimicrobial Activity Of G.O & G.P

The aim of this work was to provide evidence to test hypothesis 3. In addition, an evaluation of the composition of the surrounding medium and its influence on the antimicrobial activity of G.O, may provide an insight into its potential effectiveness within the intestinal tract and its mode of action. The results of the effects of a variety of different media are presented in Figures 3.3.3a, b.

The results show that within all the media types used (in the absence of G.O) with the exception of sodium bicarbonate, the *E. aerogenes* cells remain viable over the period of experimentation. In the presence of sodium bicarbonate it was observed that during the period of experimentation complete kill of *E. aerogenes* cells was observed. In the presence of 2.75mg/ml G.O, complete kill of the *E. aerogenes* within 240 minutes of experimentation was observed for all media types with the exception of TSB, 0.9% saline and phosphate buffer.

In terms of the effect of G.O on the reduction of cell population size over the 24 hour period the following observations were made; a) in TSB, phosphate buffer and 0.9% Saline - an approximate thousand fold reduction in cell numbers and b) in MSM+glucose, SDW and sodium bicarbonate - a complete kill.

These results suggest that TSB may perhaps reduce the antimicrobial effectiveness of G.O in some way. The observed effect could be by one or a combination of enhanced dispersal and thence volatilisation and physical/chemical removal. This uncertainty is referred to again in the Discussion (p215) in light of the results obtained from studies with cysteine (Figure 3.3.3d) and HPLC data (Section 3.6.5).

Figure 3.3.3a: The Effect Of A 2.75mg/ml G.O Concentration Within Saline, Sodium Bicarbonate, SDW And Phosphate Buffer

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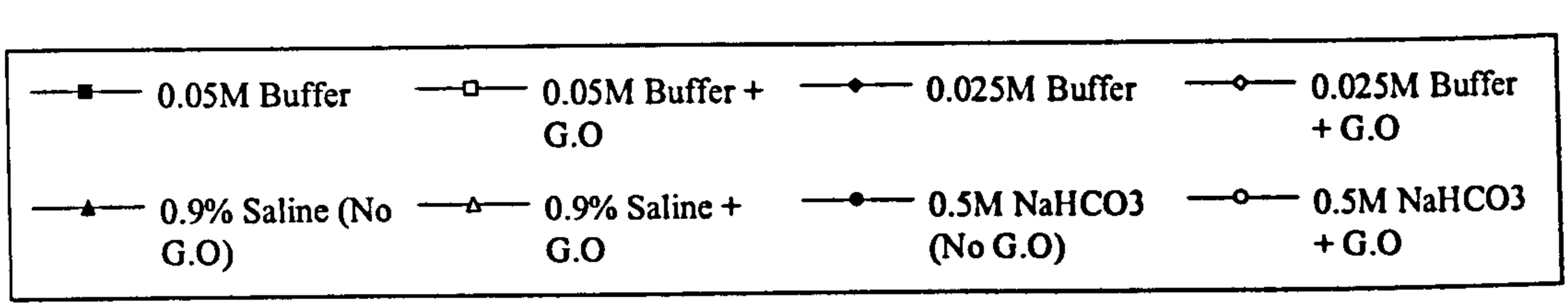

Figure 3.3.3b: The Effect Of A 2.75mg/ml G.O Concentration Within Minimal Salts Media With And Without A Carbon Source (Glucose)

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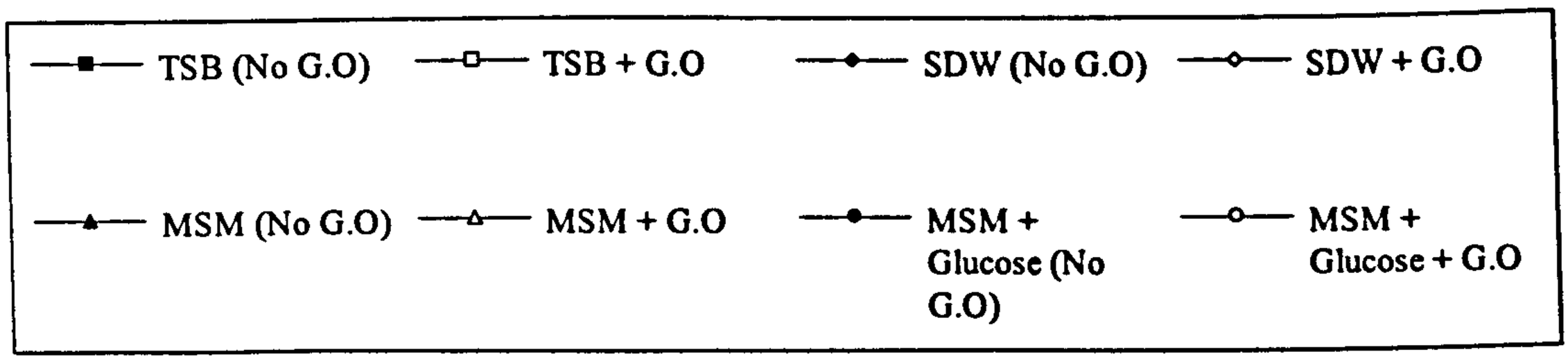


2.75mg/ml G.O concentrations were prepared in saline, sodium bicarbonate, SDW, phosphate buffer and MSM ± a carbon source (glucose) in tin foil capped boiling tubes, to a total volume of 20ml. 40µl of an overnight culture of *E. aerogenes* was used as an inoculum. Duplicate control tubes (no G.O) were set up. The tubes were incubated in a water bath at 37°C and the viability of the cells measured with respect to time using either the spread plate or Miles & Misra enumeration techniques. Comparisons of the different viability results were measured against those of TSB.

Figure 3.3.3 a

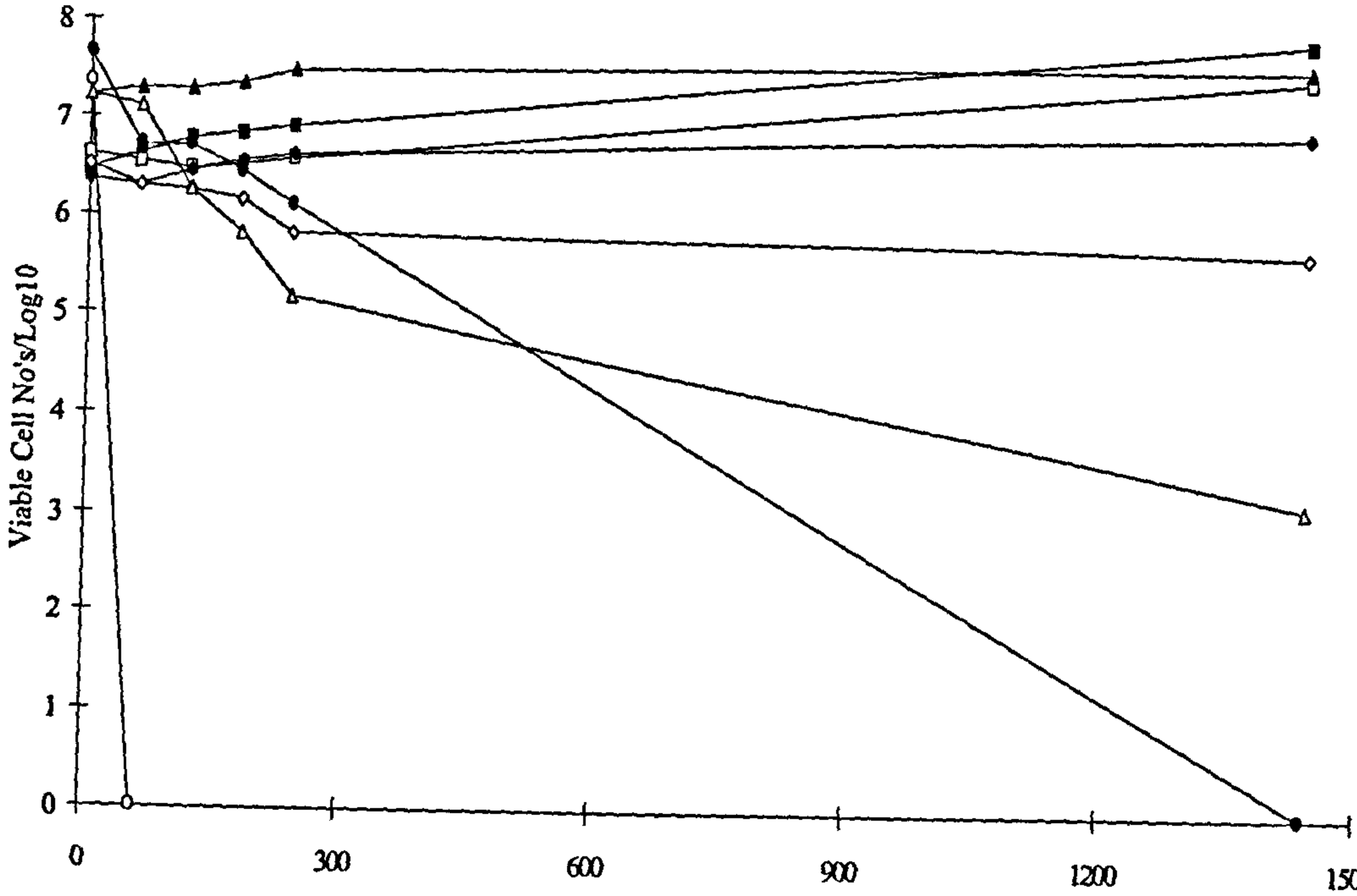
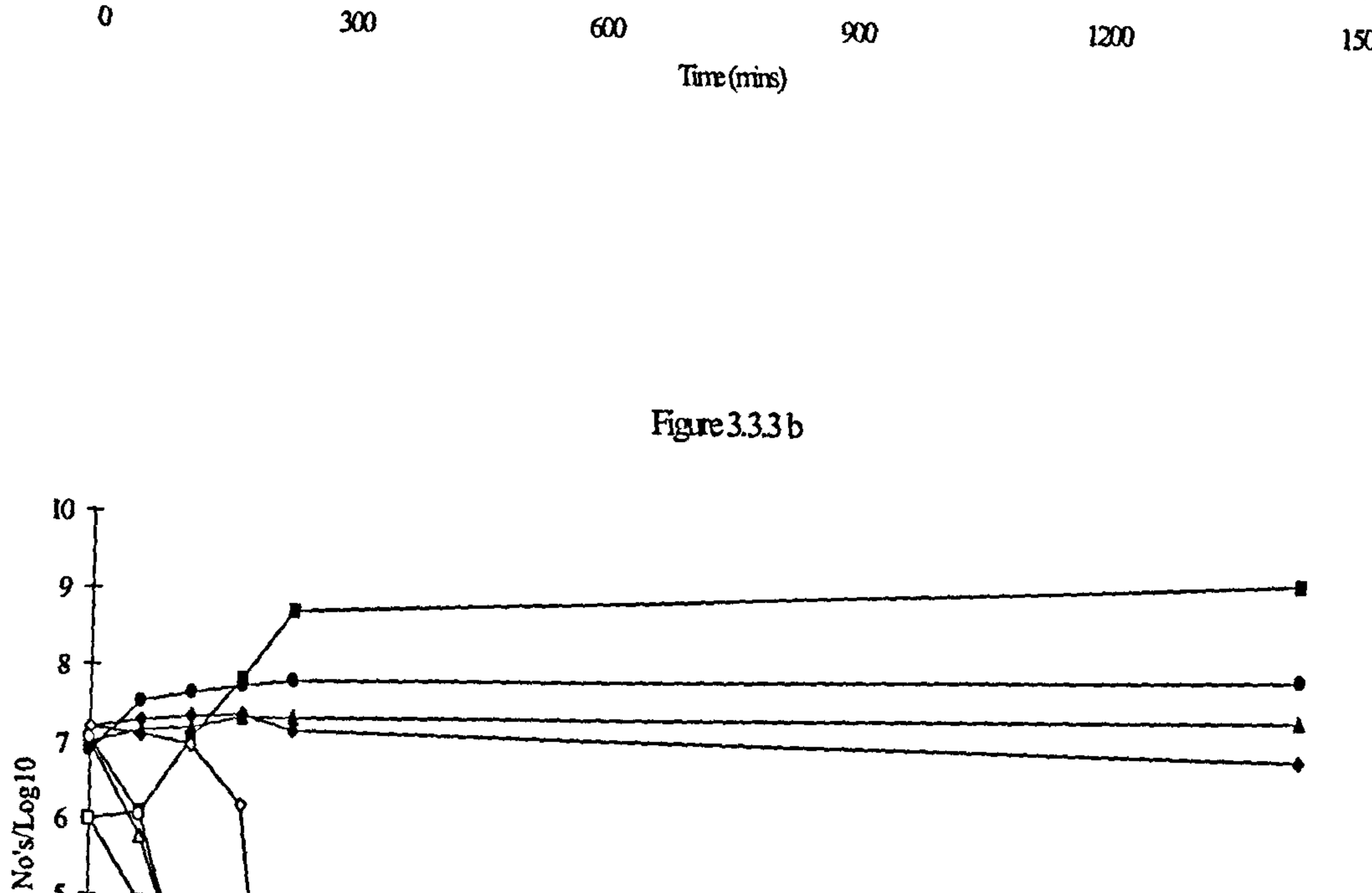


Figure 3.3.3 b



The results obtained indicated that:-

a) The presence of G.O (2.75mg/ml) in all environments results in loss of cell population viability and in the case of sodium bicarbonate, enhances the previously observed loss of viability.

b) The initial rate of cell death varied slightly according to the type of environment.

c) In all environments complete kill of the cell population was observed within 300 mins, except in TSB, phosphate buffer and 0.9% saline which only exhibited partial cell population kill over a 24 hour period.

d) The effect of G.O on *E. aerogenes* in MSM+glucose (an environment which allowed cell population growth) did not significantly differ to the effect observed for *E. aerogenes* in MSM without glucose (a non-growth environment).

To elucidate the observed difference in the effect of G.O between TSB and MSM, various concentrations of tryptone were added to MSM+glucose. The results are presented in Figure 3.3.3c.

It was shown that in the absence of G.O, tryptone stimulated growth of *E. aerogenes* cells as expected. In the presence of G.O, increasing tryptone concentrations resulted in a decrease in the effectiveness of G.O such that at 15g/L tryptone the biocidal effect of G.O was removed and *E. aerogenes* growth was observed.

Since sulphydryl groups have been implicated in the mode of action of sulphides (Wills, 1956). It was hypothesised that sulphydryl groups of amino acids may be the causative effect, thus experiments were performed to determine whether this resulted from sulphydryl containing groups within the tryptone by studying the effect of L-cysteine (a sulphydryl containing amino acid). This was confirmed (Figure 3.3.3d) by finding that the presence of cysteine at mM concentrations reduced antimicrobial effectiveness, such that at 25mM cysteine, the biocidal activity of G.O was no longer evident and at higher concentrations growth occurred.

Figure 3.3.3c: The Effect Of Addition Of Various Tryptone Concentrations On A 2.75mg/ml G.O Concentration In MSM (+Glucose)

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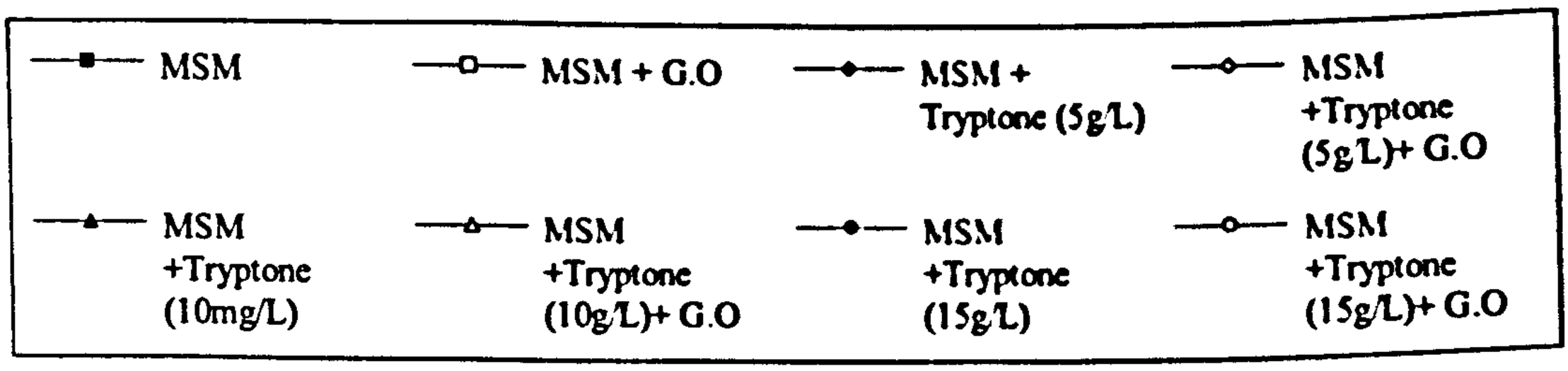

Figure 3.3.3d: The Effect Of Addition Of Various L-cysteine Concentrations On A 2.75mg/ml G.O Concentration In MSM (+Glucose)

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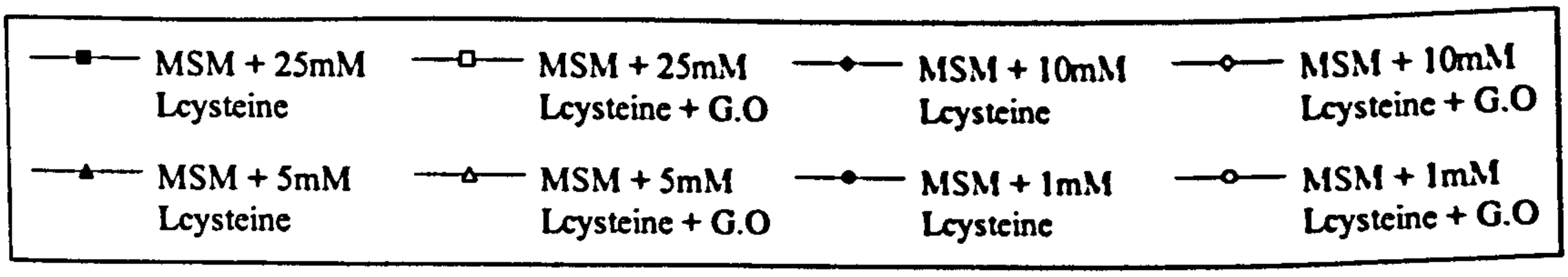


2.75mg/ml G.O concentrations were prepared in minimal salts medium with a carbon source of glucose, various tryptone and L-cysteine concentrations were also added in tin foil capped boiling tubes, to a total volume of 20ml. 40µl of an overnight culture of *E. aerogenes* was used as an inoculum. Duplicate control tubes (no G.O) were set up. The tubes were incubated in a water bath at 37°C and the viability of the cells measured with respect to time using either the spread plate or Miles & Misra enumeration techniques.

Figure 3.3.3 c

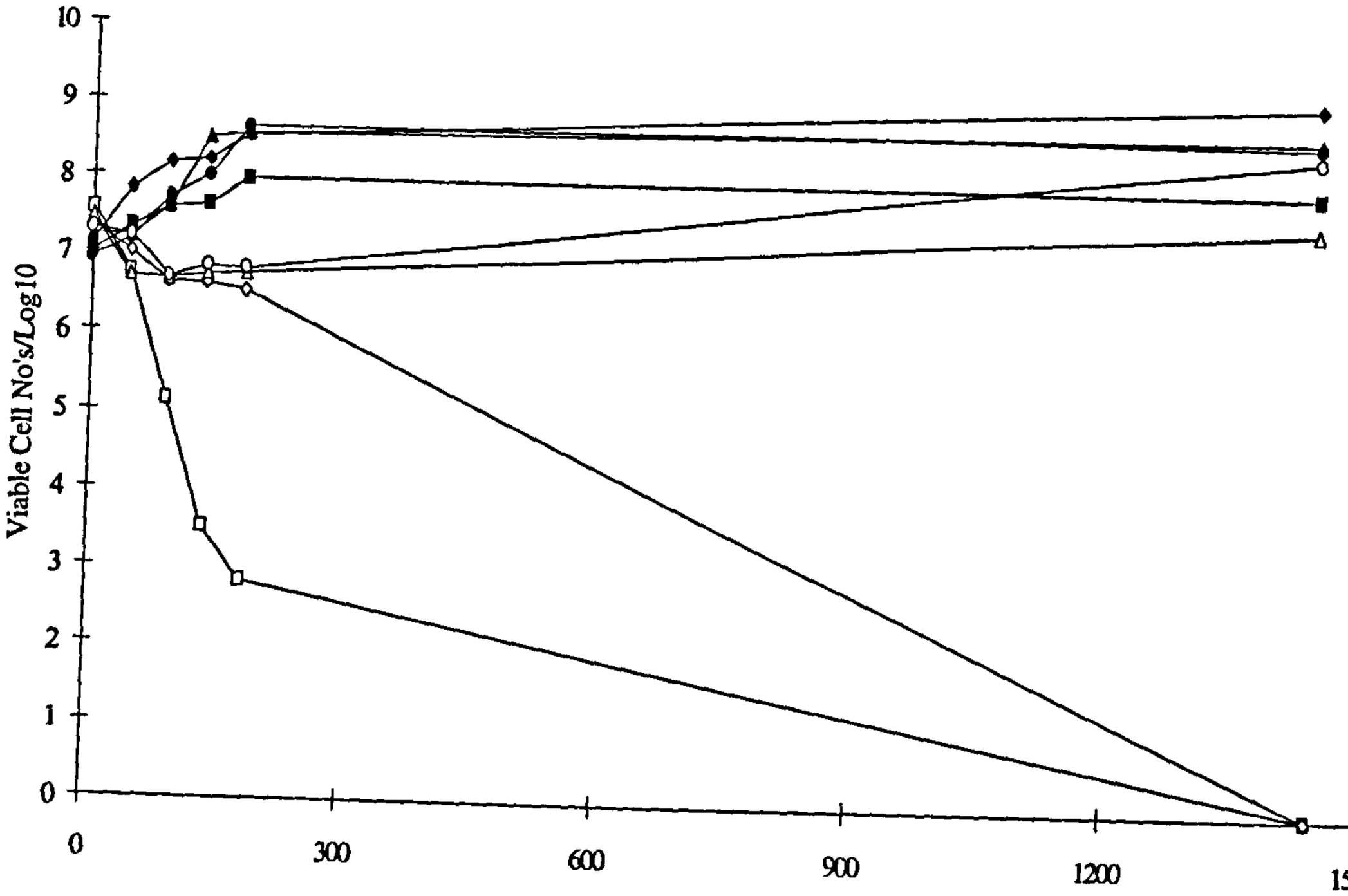
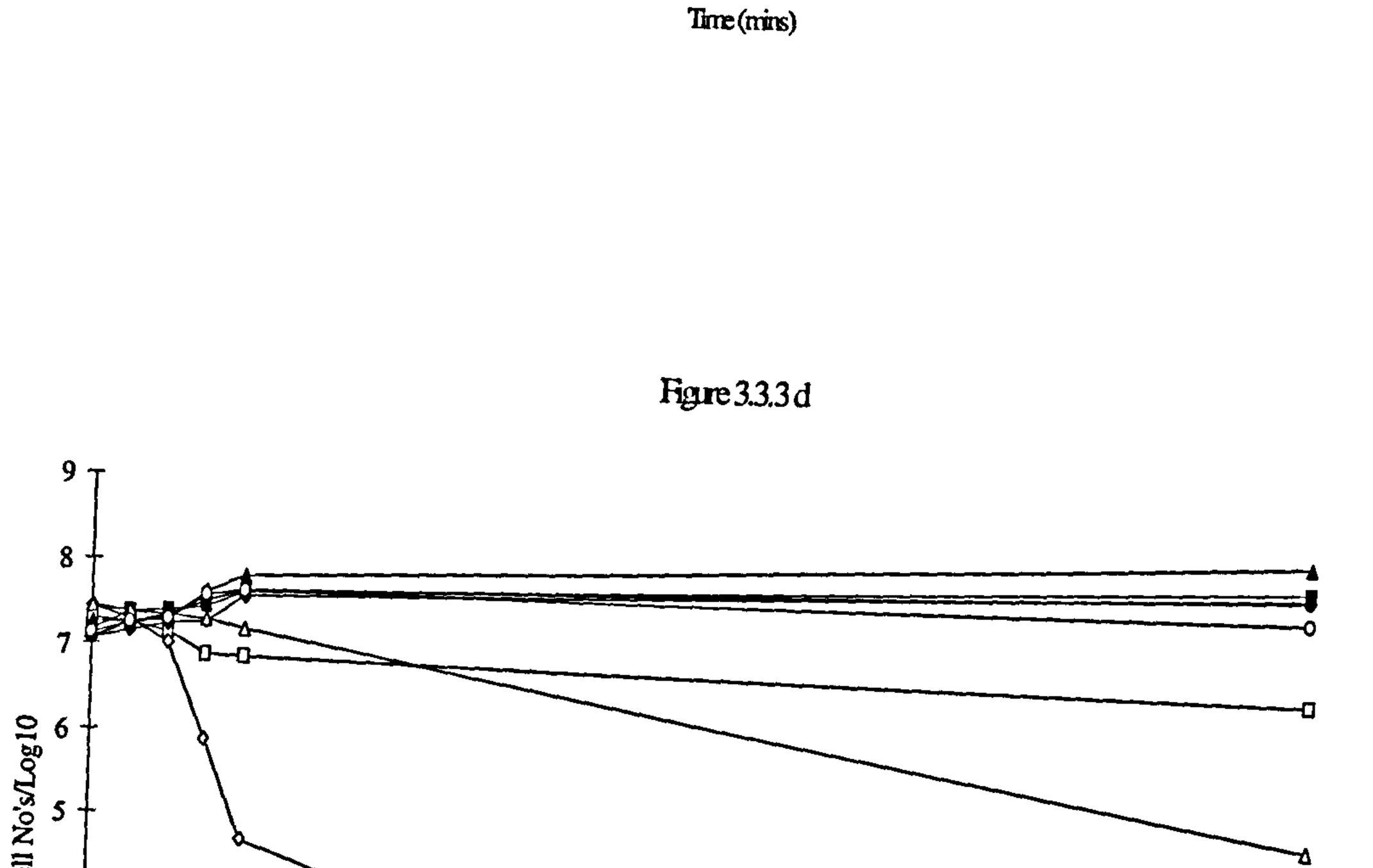


Figure 3.3.3 d



Initial studies of the effect of saline and phosphate buffer on the antimicrobial activity of G.P were performed. The results (not shown) were similar to those obtained with G.O, in that antimicrobial activity was exhibited by G.P and the extent of this activity appeared to be dependent upon concentration of phosphate buffer and saline employed. In the absence of G.P, it was shown that the *E. aerogenes* cells remain viable in the presence of saline up to concentrations of 1% and that high saline concentrations of 5% resulted in cell death. On addition of G.P (3.125mg/ml) to low saline concentrations (0.5%), it was shown that the G.P exerted a bacteriocidal effect against *E. aerogenes* after 5 hours, previous to this the cells had remained at a static population level. At the higher saline concentrations (1 & 5%), in the presence of G.P (3.125 mg/ml), the viable cell population level remained stable throughout the experiment, thus indicating a loss in the antimicrobial properties of the G.P extract.

3.3.4 The Ability Of *E. aerogenes* To Adapt To The Presence Of G.O

The aim of this experiment was to test hypothesis 4 (p88). In addition by determining whether the *E. aerogenes* cells become resistant to the antimicrobial activity of G.O with respect to time is an important consideration for the use of G.O as a therapeutic antimicrobial.

Triplicate MIC's were performed repeatedly on a daily basis over 10 days, using an inoculum which was taken from the tube showing growth at the highest G.O concentration from the previous days MIC tubes. The results are presented in Table 3.3.4a.

The results indicate that there is no evidence to support any adaptation of *E. aerogenes* to the environment. In addition there is no evidence of resistance development (over 112.9 generations) by *E. aerogenes* cell generations since the same concentration of G.O (0.68mg/ml) was required to inhibit the growth of the cells. This confirms the findings of Sashikanth *et al.* (1984) and Fulder (1990), that no resistance to the antimicrobial activity of G.O has been identified.

These results indicate that the higher MIC values recorded at 48 than 24 hours and the apparent increase in viability with time of exposure to G.O are not explicable in terms of increased microbial resistance.

Table 3.3.4a Resistance Of *E. aerogenes* To G.O With Respect To Time

Time of Incubation (Days)	MIC Value (mg/ml)
1	0.68
2	0.68
3	0.68
4	0.68
5	0.68
6	0.68
7	0.68
8	0.68
9	0.68
10	0.68

3.4 Determination Of The Antimicrobial Activity Of G.O Sulphide Components

3.4.1 Diallyl sulphide (DAS), Diallyl disulphide (DADS), Dimethyl disulphide (DMD) & Dimethyl trisulphide (DMT)

Four commercially available sulphide components of G.O were assessed for their antimicrobial activity and the results compared to those of G.O.

3.4.1.1 MIC Determinations

Initial triplicate MIC determinations were performed on *E. aerogenes* and other more sensitive bacteria under identical conditions to those performed with G.O, in order to provide a quick means of comparison of these G.O components to that of G.O (See Table 3.4.1.1a).

Table 3.4.1.1a MIC Determinations Of G.O & Various G.O Sulphides

	MIC VALUE (mg/ml)				
Organism (Culture No.)	G.O	DAS	DADS	DMD	DMT
E. aerogenes (3)	0.68	2.75	25	>10	0.31
E. coli (40)	0.68	2.75	25	>10	0.31
E. coli 055	1.37	5.5	10	>10	0.31
S. typhimurium (434)	0.34	/	25	>10	0.31
Shig. sonnei (426)	2.75	/	10	>10	0.02
L. monocytogenes (433)	0.02	11	0.63	>10	0.04
Y. enterocolytica (R6)	0.02	2.75	0.63	/	/

The 24-hour MIC results show that all four sulphide components tested displayed antimicrobial activities and that each showed substantially different antimicrobial activities from one another and from G.O. Only DMT showed consistently higher antimicrobial activity than G.O, which accounts for only around 4% of G.O (as determined by GC analysis, Section 3.6.2). HPLC analysis purity checks (See Section 2.1.6.3.2) indicated that the separate sulphides obtained were significantly contaminated with other garlic sulphides. This seemed to indicate that the antimicrobial activities measured might not be more than approximations. In practice the 'separate' sulphides produced MIC values substantially different from one another. This suggests that the values obtained probably characterise the antimicrobial activities of each sulphide fairly accurately. The antimicrobial activity observed with each separate sulphide suggests that together these constituents can explain all or much of the antimicrobial action of G.O.

Comparisons of DAS and DADS showed that, in most cases, DAS has substantially greater antimicrobial activity than DADS against most of the organisms tested. This is in agreement with the recently published data of Lawson *et al.* (1991a). In this study *L. monocytogenes* and *Yersinia enterocolytica* were exceptions to this pattern, displaying an especially high sensitivity to DADS in comparison with other species. The results

indicate that although both sulphides display antimicrobial activity, neither are as effective as the complete G.O. This is peculiar, since the DADS and DAS components account for 30 and 3% (as identified from GC analysis, Section 3.6.2) respectively, of the total G.O. Hence the greater antimicrobial effects of G.O may be a consequence of synergistic effects between G.O sulphides present, or alternatively it may result from the antimicrobial action of other untested sulphides, or some so far unidentified substances within the G.O. Another possible reason is that volatilisation rates differ between G.O and "pure" sulphides in the milieu used (See p215 for a more detailed discussion). The low MIC value recorded for DMD may also result from such an effect, as it is one of the most volatile sulphide components of G.O (B.pt 109.6°C).

3.4.1.2 The Effect Of Various Concentrations Of DAS & DADS On *E. aerogenes* Viability

The aim of these experiments were to compare the effect upon cell viability of DAS and DADS to that of G.O. The results are presented in Figures 3.4.1.2a, b.

The initial effect of DADS on *E. aerogenes* viability is similar to that of G.O. However the 0.62-5mg/ml concentrations tested are all associated with an initial decline in population size. After this 2 hour period an increase occurs which could result from the effects such as those hypothesised in Section 3.2 (p88). It should be noted, that the MIC for *E. aerogenes* in TSB+DADS was determined as 25mg/ml which is higher than the concentrations of DADS used in this experiment.

These differences could be due to a number of possibilities; 1) MIC determinations are performed in close fitting capped test tubes while in viability experiments only tin foil capped boiling tubes are used OR 2) Differences in experimental technique, such as the repeated disturbance of the tubes for sampling purposes in the viability study. Thus greater loss by volatilisation of antimicrobial components to the atmosphere is likely to occur during the viability studies than during the MIC determinations.

The effect of DAS on *E. aerogenes* viability through time, showed that low concentrations (0.62-1.25mg/ml) have no significant effect on *E. aerogenes* cell population growth and viability as similar results to the control (without DAS) were observed. At the higher concentrations of 2.5 and 5mg/ml, an initial decline in the viable population occurs, after which cell population increases are observed and as with G.O (concentration 1.375mg/ml, Figure 3.2a), this commences sooner with lower concentrations (Figure 3.4.1.2a).

A similar pattern of results is seen with the different concentrations of DADS tested, although DADS was more potently antimicrobial than DAS at all concentrations. This difference is most noticable early on and is reflected in the initial cell death rates (Figure 3.4.1.2c). These results are the reverse of the MIC results in which DADS was substantially less antimicrobial than DAS for *E. aerogenes*. This apparent discrepancy may be a result of more rapid volatilisation of DAS than DADS in the viable count procedure. This is suggested by the earlier recovery of growth at 2.5 and 5mg/ml DAS than for the corresponding DADS concentrations. It is feasible because of the frequent mixing and sampling necessary in the viable count procedure and the lower boiling point of DAS (B.pt 138°C) than DADS (B.pt 180°C).

Comparison of the apparent "MIC" for DAS in the viability study with the MIC proper shows that comparable values are obtained. The MIC error could be x2 (for example 5.5mg/ml) corresponding to the viable count difference or could be 1.375mg/ml which is only slightly (but not significantly) different from 1.25mg/ml used in the viability studies. The corresponding comparison for DADS shows a significant difference between the viable count "MIC" and the true MIC. It may also be that increased mixing frequency early on in the viable count time-period promotes contact between the DADS and the bacterial cells in a way that merely seems to increase the loss by volatilisation of DAS. No direct evidence is available to resolve this difference however.

Comparison of DAS and DADS (Figure 3.4.1.2c) to that of G.O (Section 3.2, Figure 3.2a) indicates similarities in the rate and level of initial cell population death in which both cases is product concentration dependent. The increase in cell population size at 24 hours observed for all concentrations of DADS and DAS is explicable in terms of the hypotheses mentioned on p88.

Figure 3.5.1b: Comparison Of Viability Studies For *E. coli* (40) In TSB, CIF & SIF

Control viability studies (no G.O) were performed in; 1) TSB a biological growth medium; 2) CIF a complex simulated intestinal fluid AND 3) SIF a simple simulated intestinal fluid.

40µl of an overnight culture of *E. coli* (40) was added to each of the three media types of a total volume of 20ml in tin foil capped boiling tubes. The tubes were incubated at 37°C in a water bath and the viability of the cells measured with respect to time using either the spread plate or Miles & Misra enumeration techniques.

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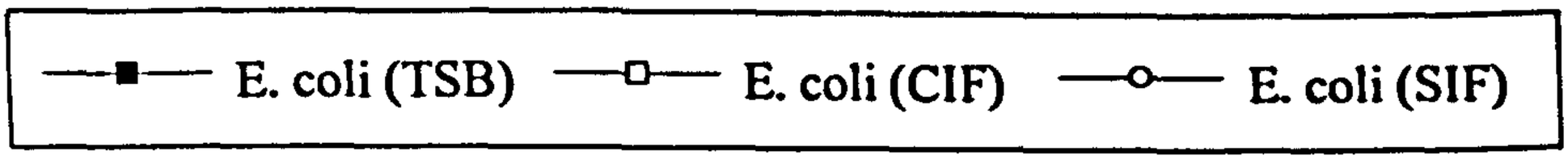

Figure 3.4.1.2 a

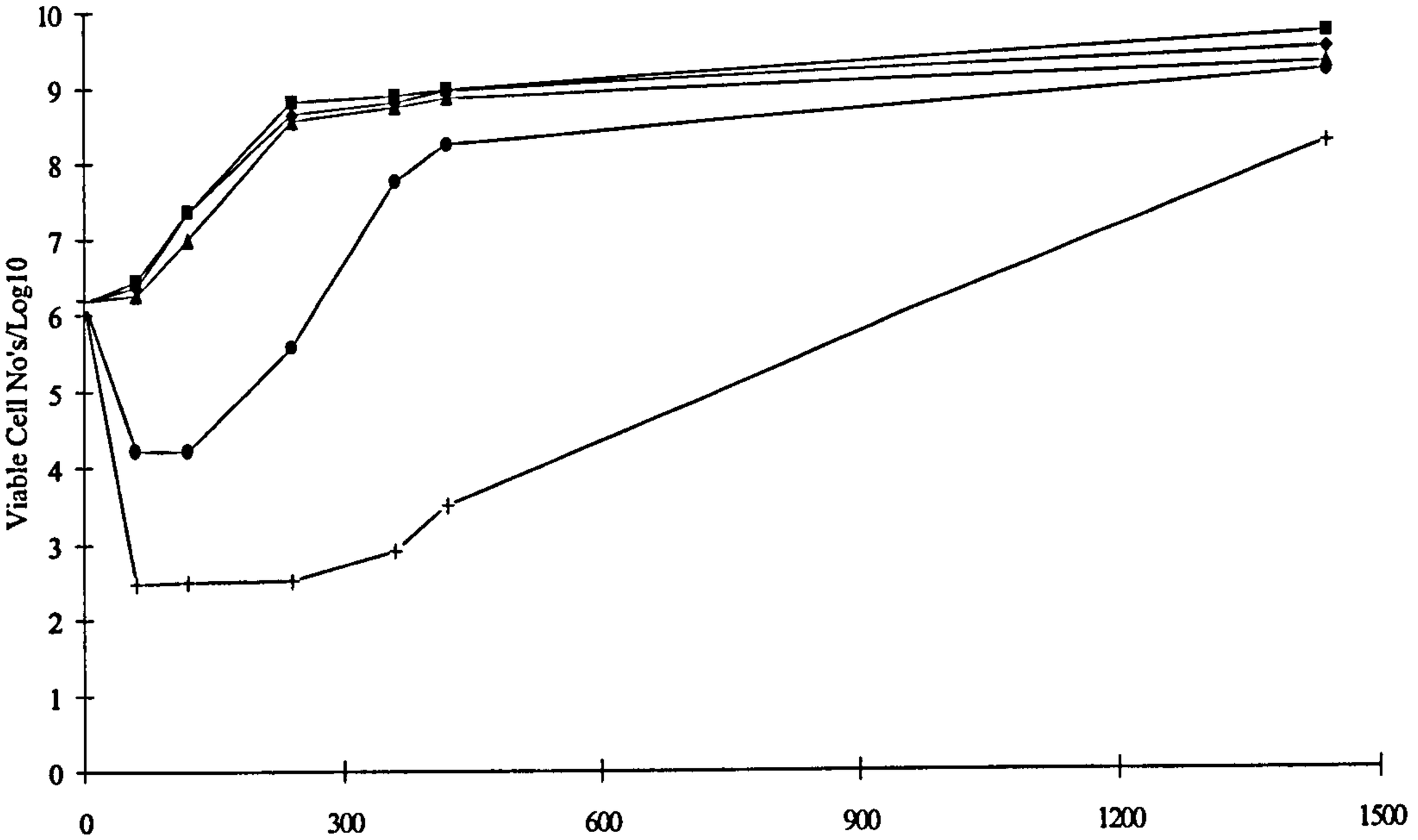

Figure 3.4.1.2 b

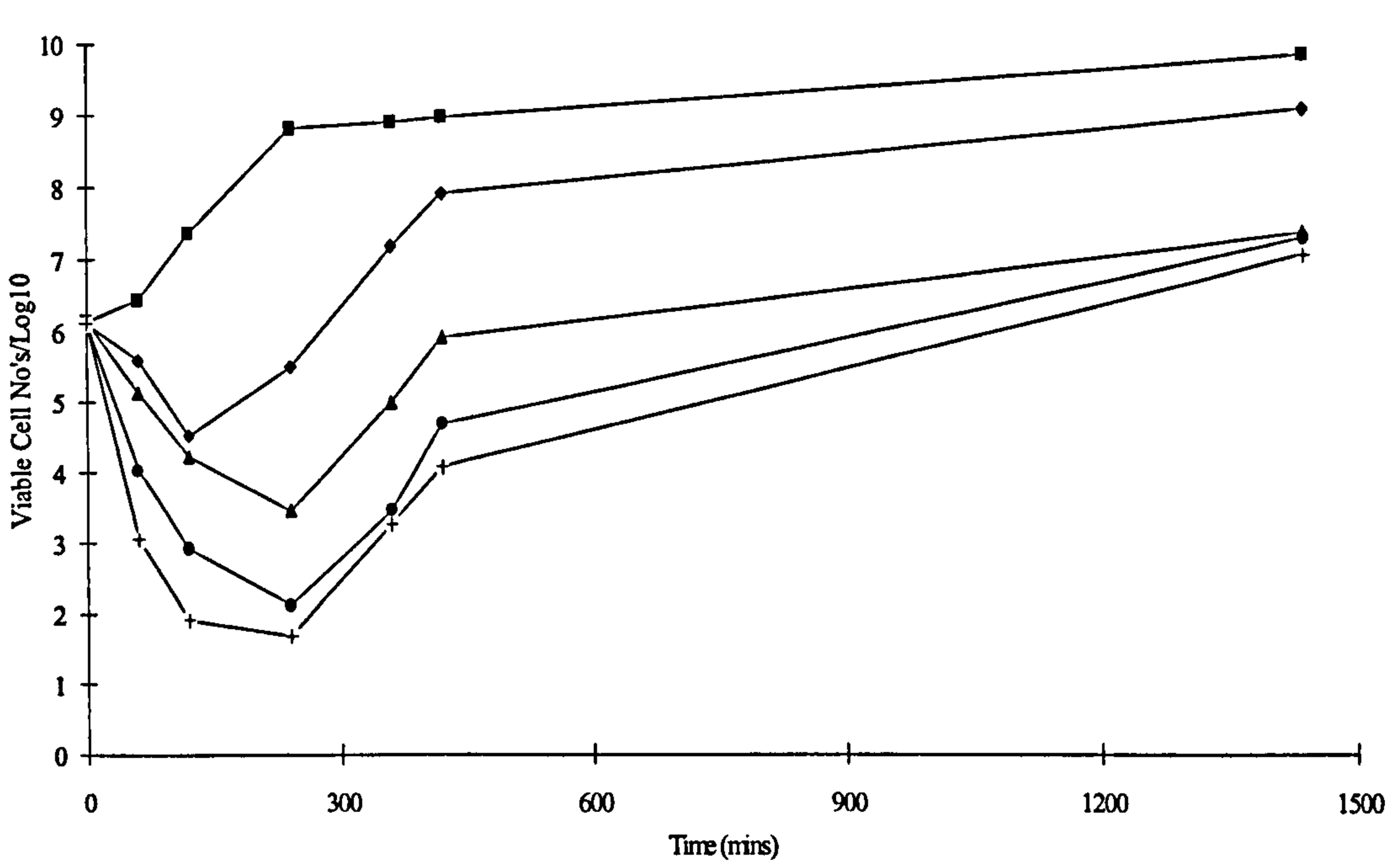

Figure 3.4.1.2c: <u>The Effect Of Diallyl disulphide (DADS) & Diallyl sulphide (DAS) On Initial Cell Death/Growth Rates</u>

The source of data for these graphs was derived from the initial cell death/growth rates determined from Figures 3.4.1.2a & b.

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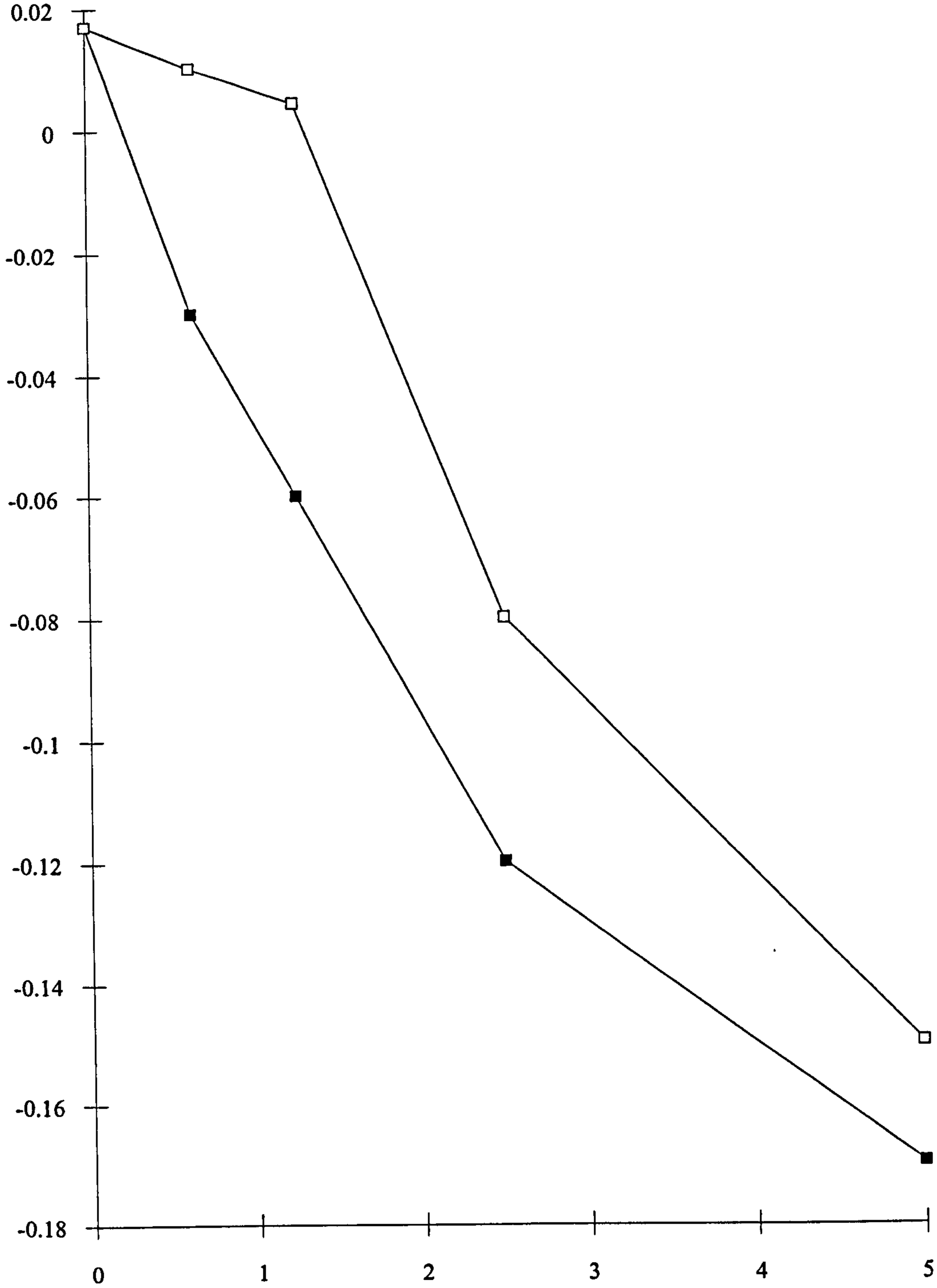
Figure 3.4.1.2 c



3.5 The Ability Of G.O To Exert Antimicrobial Activity Within Intestinal Fluid Models

3.5.1 The Effect Of G.O On Selected Enteric Organisms Within The Simulated Gut Fluids Simple Intestinal Fluid (SIF) & Complex Intestinal Fluid (CIF)

The aim of these experiments was to compare the antimicrobial activity of G.O in TSB (Section 3.1) to that seen within simulated intestinal environments. Five enteric organisms were chosen for these studies, three of which are known enteric pathogens. MIC tests in triplicate (Table 3.5.1a) and repeated viability studies utilising *E. coli* (40) were performed in the two simulated gut fluid models; SIF (simple) and CIF (complex), first without (Figure 3.5.1b), and then with G.O (Figures 3.5.1c-e).

Table 3.5.1a MIC Determinations Of G.O

	MIC Value (mg/ml)		
Organism (Culture No.)	TSB	CIF	SIF
E. aerogenes (3)	0.68	0.68	2.75
E. coli (40)	0.68	0.34	0.34
L. monocytogenes (433)	0.02	0.01	0.04
S. typhimurium (434)	2.75	0.34	0.34
Shig. sonnei (426)	2.75	0.34	0.08

Comparison of the MIC's obtained shows that the medium environment is important to the extent of G.O antimicrobial activity seen. This confirms the value of seeking a "model" medium as closely comparable to the *in vivo* environment as possible. The relatively simple intestinal fluid (SIF) gave results that were inconsistent in comparison with the complex intestinal fluid (CIF). This may reflect the very different and nutritionally incomplete nature of SIF as compared to the other two media. In contrast CIF provided MIC results which were consistently lower (or the same, *E. aerogenes*) than for TSB. The investigations indicating that tryptone SH-groups in

TSB effectively "neutralise" garlic sulphides may explain the apparently greater sensitivity to G.O seen in CIF as compared to TSB.

The viability studies showed that growth within CIF was similar to that in TSB, whereas within SIF, the initial period of 60 minutes showed a decline in the *E. coli* population, so that the final population size is lower than that observed in both CIF and TSB.

Figure 3.5.1b: Comparison Of Viability Studies For *E. coli* (40) In TSB, CIF & SIF

Control viability studies (no G.O) were performed in; 1) TSB a biological growth medium; 2) CIF a complex simulated intestinal fluid AND 3) SIF a simple simulated intestinal fluid.

40µl of an overnight culture of *E. coli* (40) was added to each of the three media types of a total volume of 20ml in tin foil capped boiling tubes. The tubes were incubated at 37°C in a water bath and the viability of the cells measured with respect to time using either the spread plate or Miles & Misra enumeration techniques.

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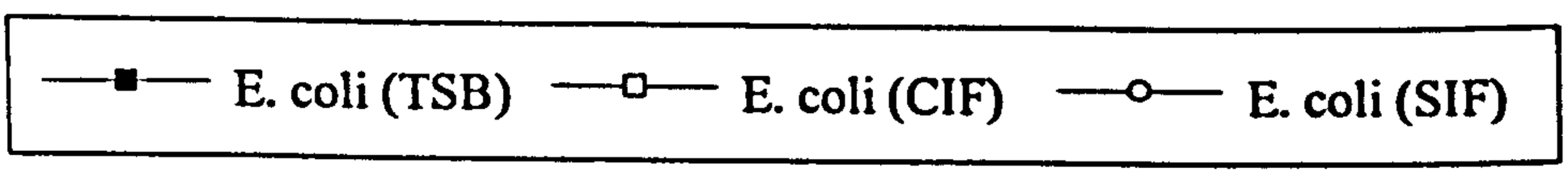
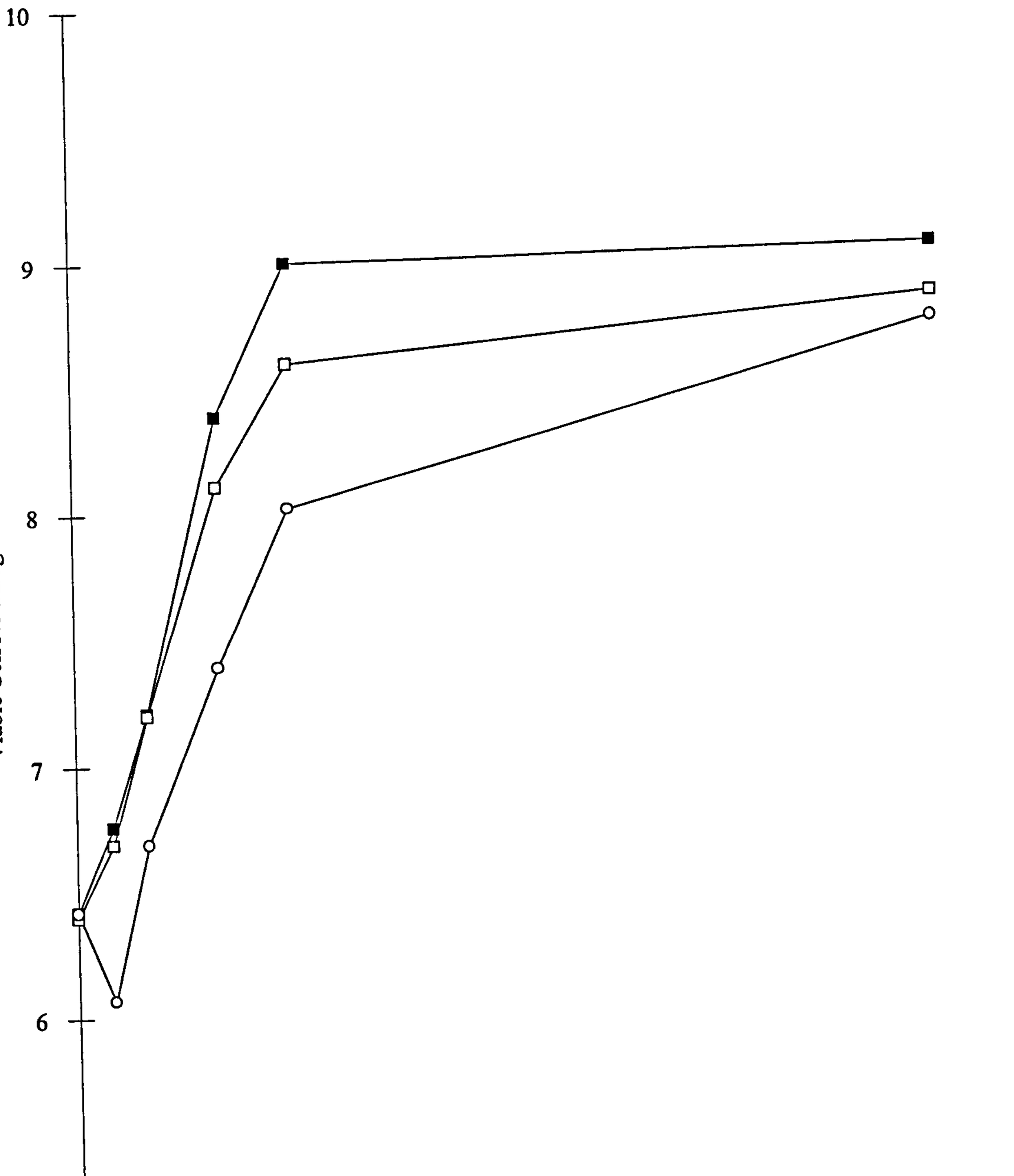


Figure 3.5.1 b


10
9
8
7
6
5
Viable Cell No's/Log10
0
300
600
900
1200
1500
Time (mins)

The effect of various G.O concentrations on *E. coli* (40) within CIF and SIF were also assessed in comparison with TSB.

In all of TSB, CIF and SIF (Figures 3.5.1c-e) the apparent "MIC" from the viability studies are all within the error range (that is a factor of x2 either way) of those determined by the MIC method. The results in SIF produced a less coherent pattern than in the other media. Thus the G.O concentration versus viability effect was not well-graded, and except at the highest concentration observed sensitivity to G.O was less than in either TSB or CIF.

In contrast, as for the MIC results, both TSB and CIF provided comparable results with CIF associated with slightly greater sensitivity of *E. coli* (40) to G.O at all concentrations used.

Figure 3.5.1c: The Effect Of Various G.O Concentrations On *E. coli* (40) In TSB

Figure 3.5.1d: The Effect Of Various G.O Concentrations On *E. coli* (40) In CIF

Figure 3.5.1e: The Effect Of Various G.O Concentrations On *E. coli* (40) In SIF

Serial two-fold dilutions of G.O were prepared in TSB, CIF and SIF in tin foil capped boiling tubes, to a total volume of 20ml. 40µl of an overnight culture of *E. coli* (40) was used as an inoculum. The tubes were incubated in a water bath at 37°C and the viability of the cells measured with respect to time using the Miles & Misra enumeration technique.

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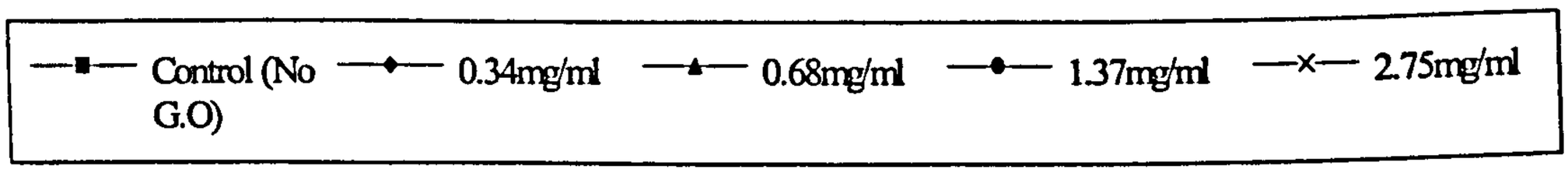


Figure 3.5.1 c

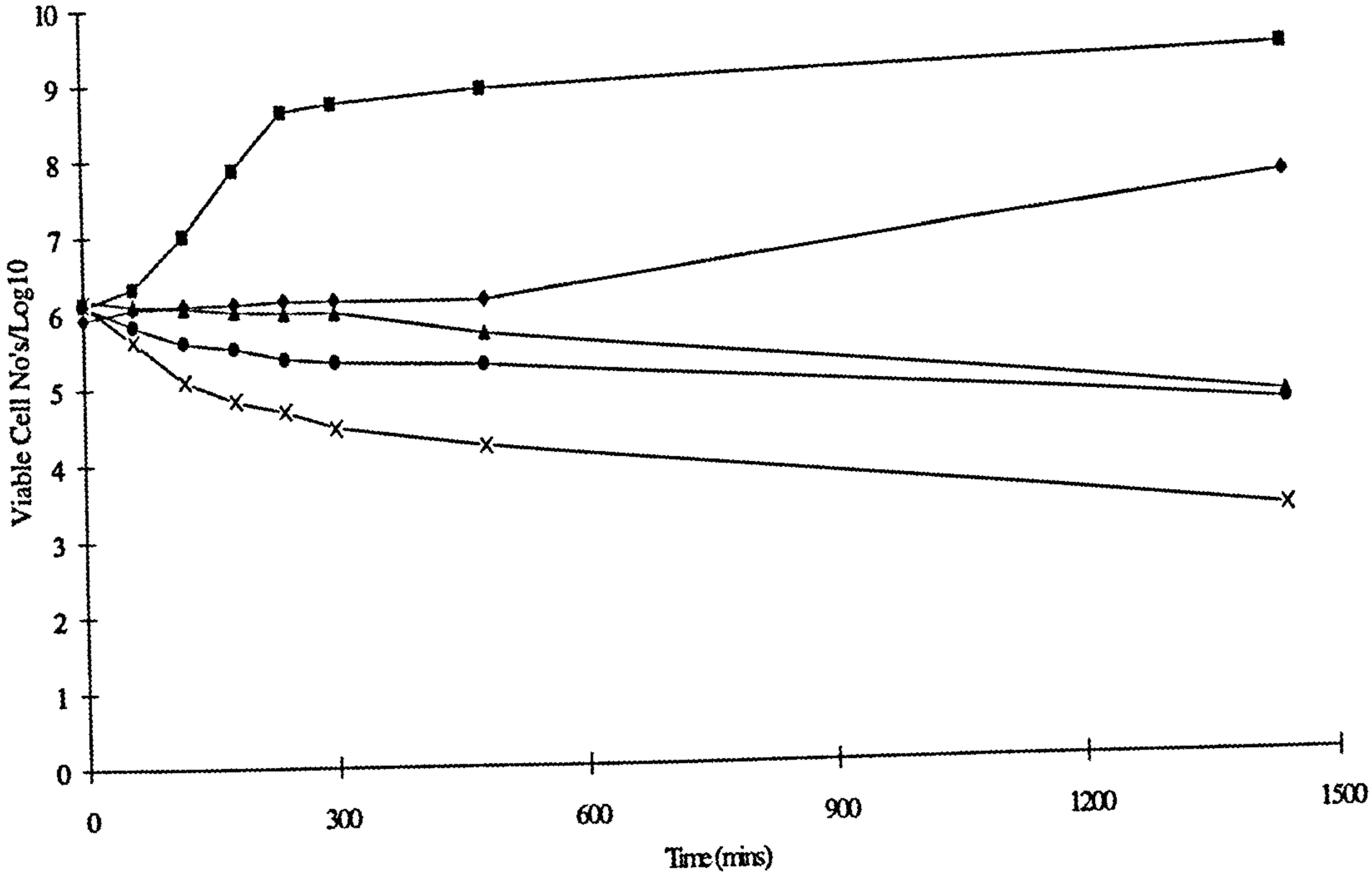


Figure 3.5.1 d

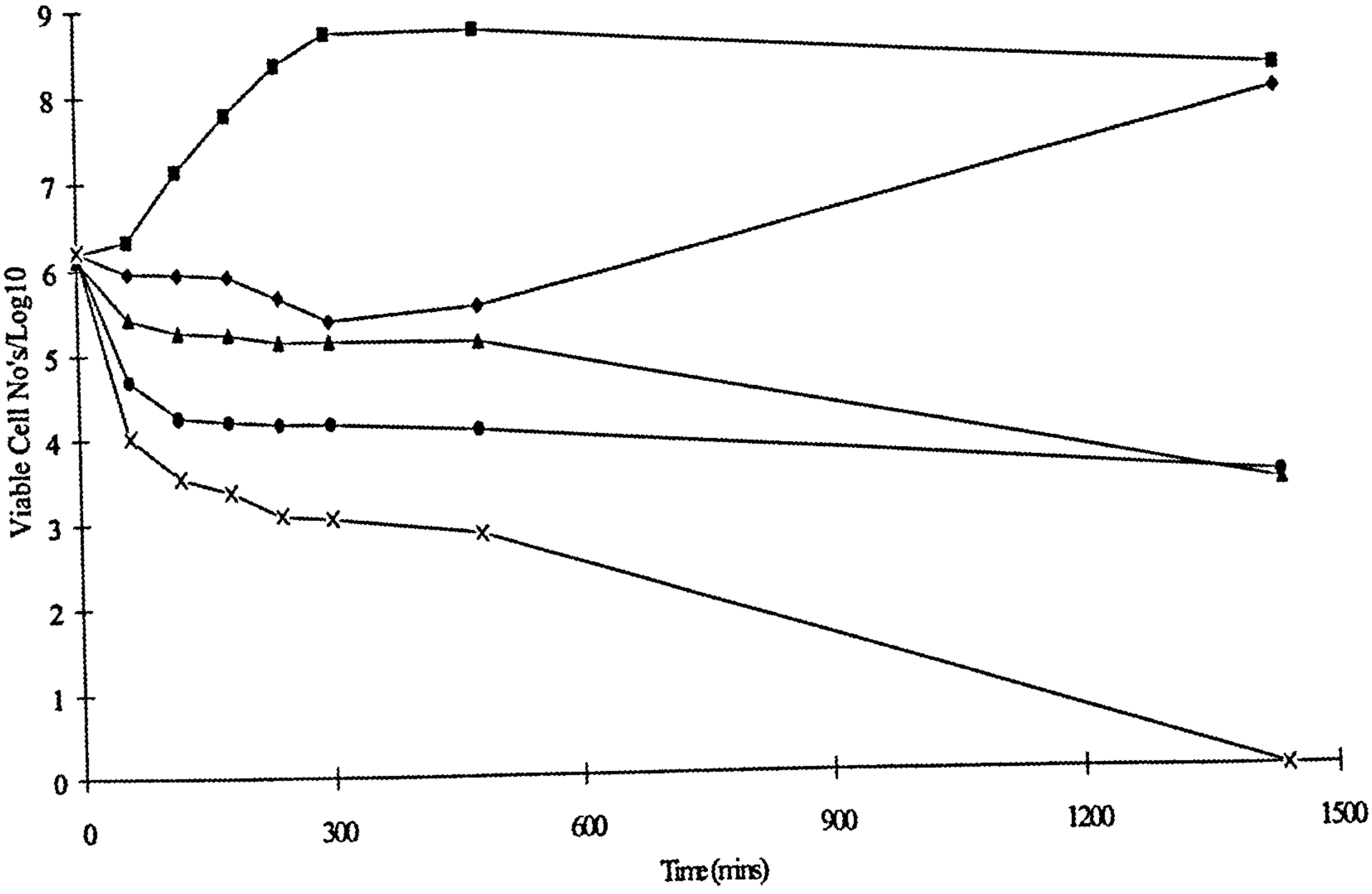
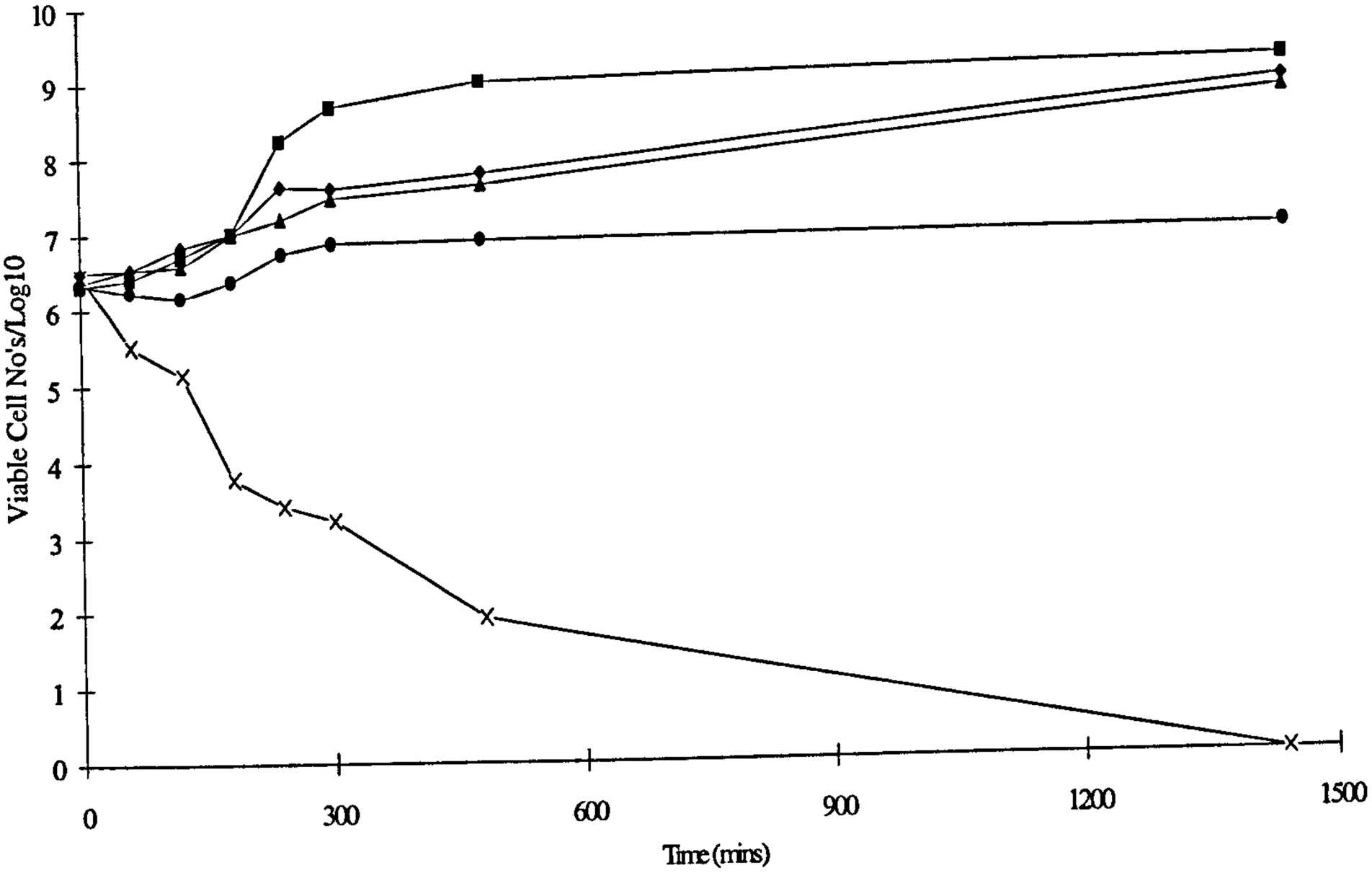


Figure 3.5.1 e


Viable Cell No's/Log10
10
9
8
7
6
5
4
3
2
1
0
0
300
600
900
1200
1500
Time (mins)

Comparison of the initial cell death/growth rates in the three media tested with different levels of G.O (Figure 3.5.1f) show that a higher concentration of G.O was required to completely inhibit growth in TSB than in CIF although the rates were generally similar. The presence of G.O in SIF, however does not appear to have any effect on the *E. coli* cells until at a G.O concentration greater than 1.37mg/ml.

The results obtained both from the MIC and viability studies with SIF were probably a consequence of its simple composition (phosphate buffer, pancreatin, sodium hydroxide and distilled water). Thus may also explain the relatively unpatterned effect of G.O in SIF upon the initial cell death/growth rates (Figure 3.5.1f). Because of this and its more suitable modelling of the gut environment CIF was preferred to both TSB and SIF in subsequent investigations of G.O antimicrobial activity.

Figure 3.5.1f: Comparison Of Initial Cell Death/Growth Rates For *E. coli* (40) In TSB, CIF & SIF

The source of data for this graph was derived from the initial cell death/growth rates determined from Figures 3.5.1c-e.

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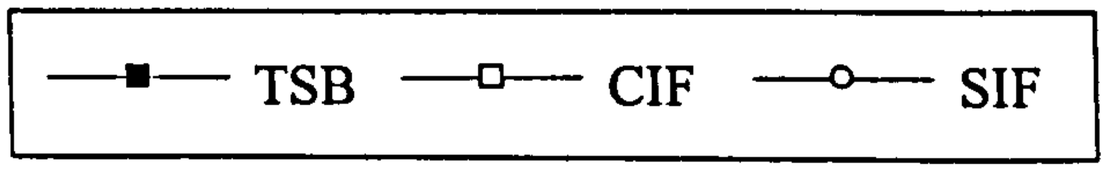
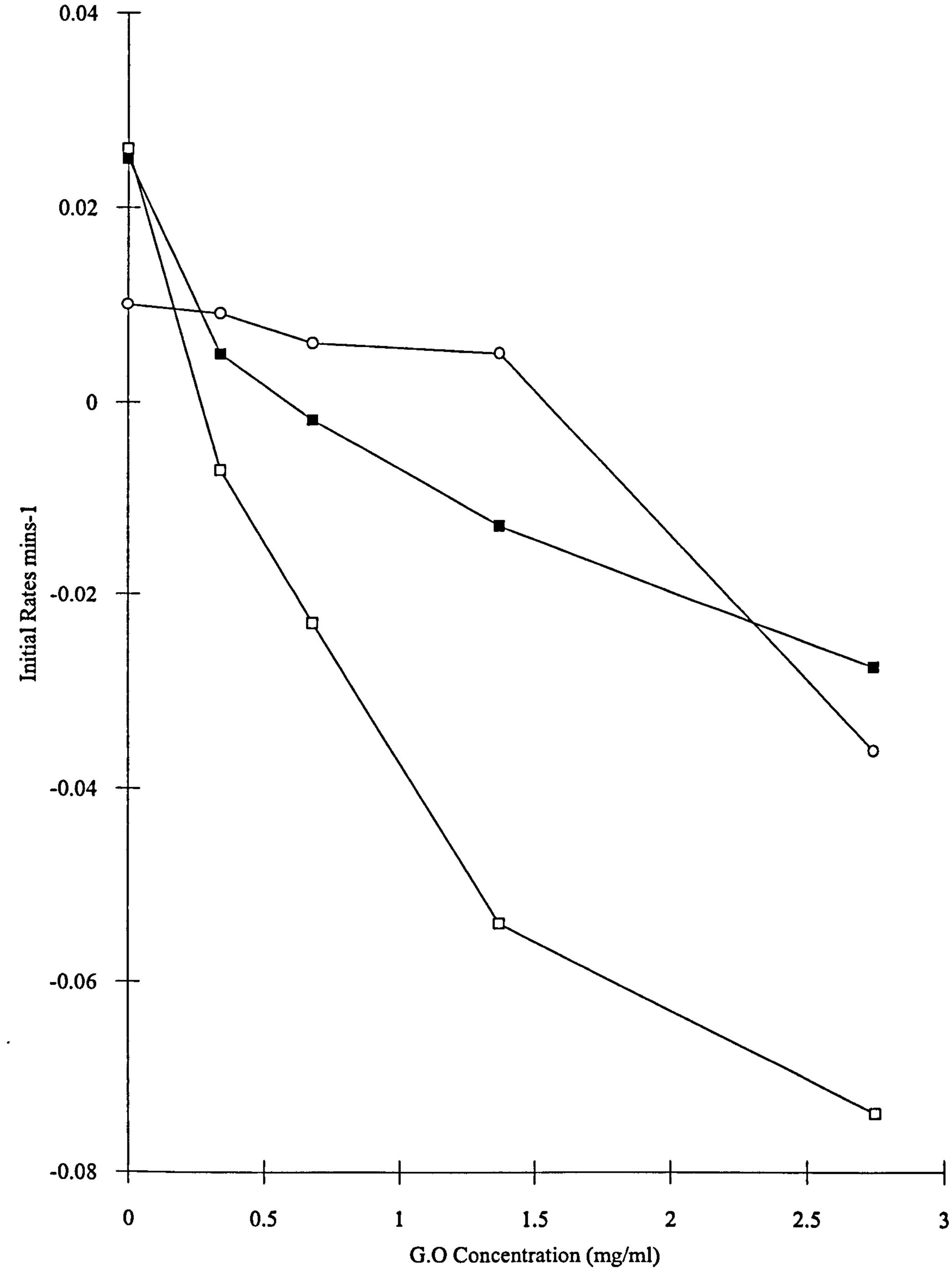


Figure 3.5.1f


0.04
0.02
0
-0.02
-0.04
-0.06
-0.08
Initial Rates mins-1
0
0.5
1
1.5
2
2.5
3
G.O Concentration (mg/ml)

3.5.1.1 Further Evaluation Of The Influence Of CIF Upon Antimicrobial Activity Of G.O

To further compare the effects of G.O within TSB with that of CIF, in order to evaluate the effect of simulated gut fluids as an environment on the antimicrobial activity of G.O, further pure culture viability studies in the absence of G.O were initially performed in both CIF and TSB on three selected intestinal pathogens (Figure 3.5.1.1a). These were performed on three different occasions in order to provide valid reproducible results. The reasoning behind this work was; 1) TSB was the only growth medium regularly used in viability studies; 2) preliminary studies with other growth media (MSM+glucose, Figure 3.3.3b) indicated differences in the G.O antimicrobial effectiveness can occur; 3) TSB was found to contain an inhibitor to G.O antimicrobial activity AND 4) if other media are to be used, a suitable simulated gut fluids such as CIF is clearly amongst the most relevant.

In all cases (except *L. monocytogenes* and *Shig. sonnei* in CIF), growth and viability of the enteric organisms investigated is similar in both media types. The initial decline in both *L. monocytogenes* and *Shig. sonnei* populations within CIF, may be due to the presence of inhibitory components within the medium (such as bile at a concentration of 0.56%). It is known that both *L. monocytogenes* and *Shig. sonnei* are sensitive to bile, *Shigella* being the more sensitive (Mabey, Personal Communication). Figure 3.5.1.1a indicates, that *L. monocytogenes* is more sensitive than *Shigella* in CIF, having a slower initial growth rate over the first 60 minutes. Over the 24 hours, it can be seen that both organisms overcome this initial decline in population reaching a considerable final population size, however it was observed that the size of the final population is greatly reduced when compared with final population levels of both organisms in TSB, which appears to support the theory of sensitivity towards the presence of an inhibitory component.

Figure 3.5.1.1a: Comparison Of Viability Studies In TSB & CIF

The four organisms used in this investigation were: *E. coli* (40), *S. typhimurium* (434), *L. monocytogenes* (433) and *Shig. sonnei* (426).

40µl of the overnight culture of each organism was used to inoculate the two media types of a total volume of 20ml in tin foil capped boiling tubes. The tubes were incubated at 37°C in a water bath and the viability was measured with respect to time using either the spread plate or Miles & Misra techniques.

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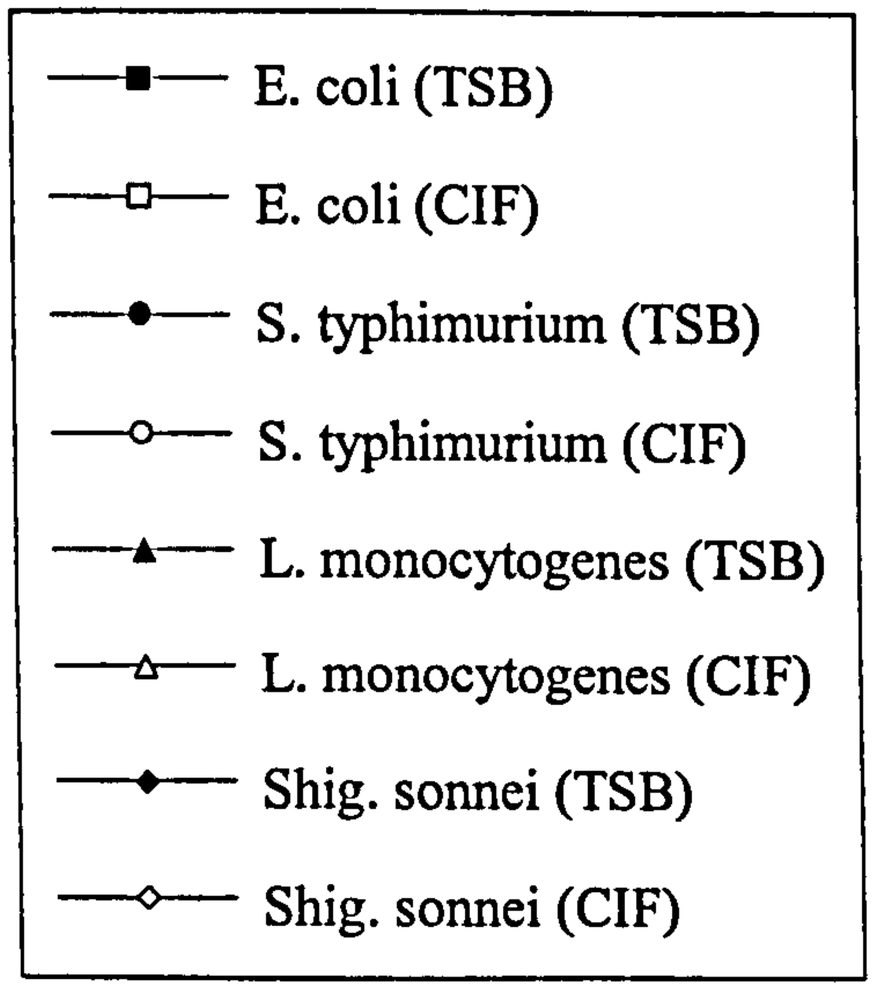
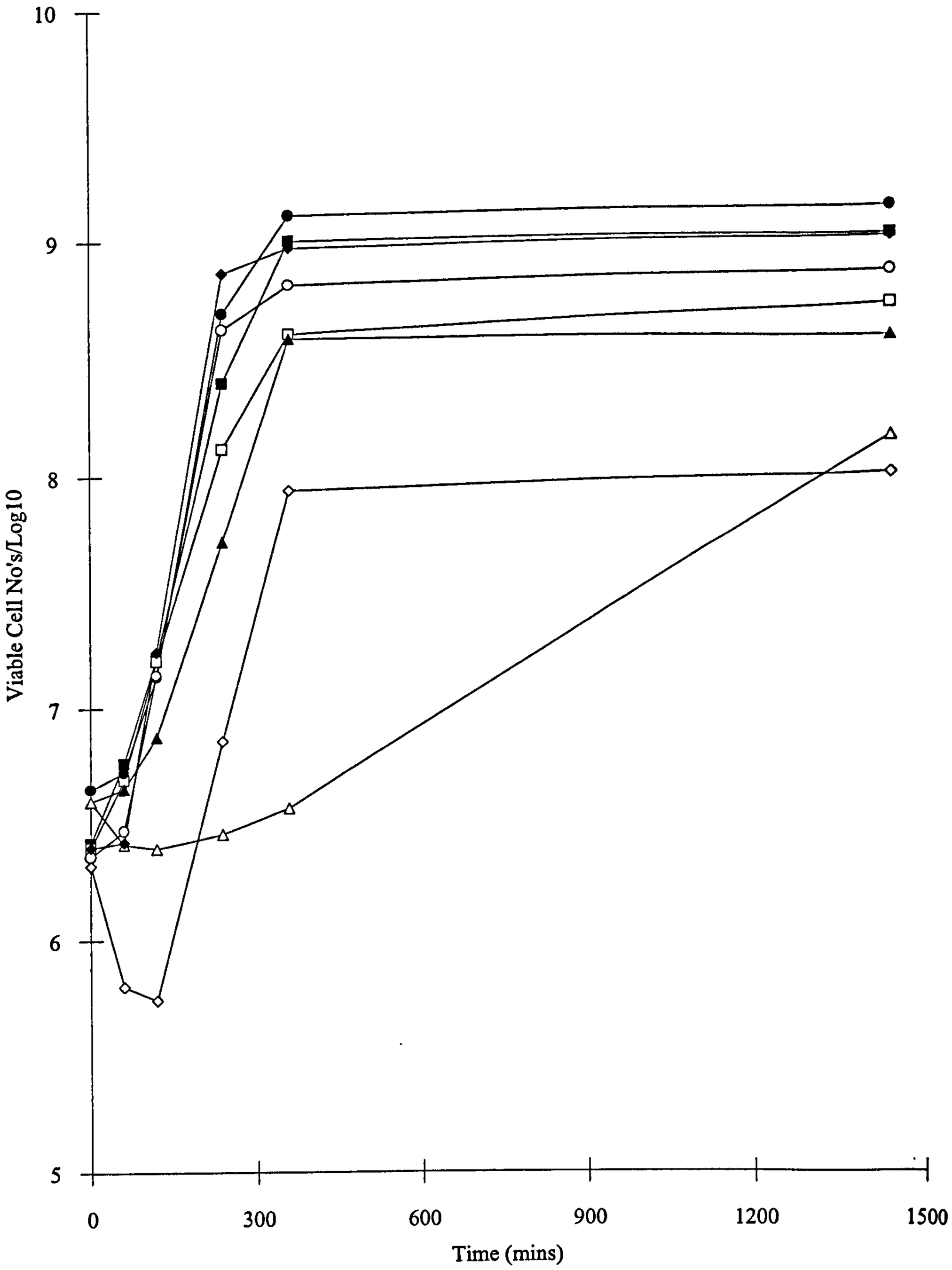


Figure 3.5.1.1 a



The effect of G.O on the viability of the selected organisms in CIF was then investigated (on numerous occasions) and compared to that in TSB. The results are presented in Figures 3.5.1(c, d) and 3.5.1.1b-g.

It was noted that some of the "MIC" values obtained from these viability studies were different from those expressed in Table 3.5.1a.

The results obtained with *S. typhimurium* indicate the similarities in response of this organism to the activity of G.O within the two media types, where the presence of G.O in both TSB and CIF produced distinct phases of cell population response. Thus at 0.34mg/ml the phenomenon of a 'lag' phase before the bacteria start to proliferate is seen in both media. Also post initial reductions in growth rates (0.68-2.75mg/ml), all of which appear to be proportional to the concentration of G.O present, occur in both media. The apparent "MIC" from the viability studies is within the two fold error of the CIF MIC proper, however that for TSB (0.68mg/ml) is four times lower than the recorded MIC (2.75mg/ml).

Initial observations indicated that both *L. monocytogenes* and *Shig. sonnei* appear to be relatively sensitive to the G.O within CIF (compared with *S. typhimurium*) and a lower concentration of G.O (0.68 and 0.34mg/ml respectively) was required to either kill or inhibit their growth (compared to that required in TSB, 0.68 and 1.37mg/ml respectively). As with *S. typhimurium*, it was observed that reductions in growth rates of both *L. monocytogenes* and *Shig. sonnei* were proportional to the concentration of G.O (0.08-0.68mg/ml and 0.17-2.75mg/ml respectively), however it should be noted that the *L. monocytogenes* cells do not grow in CIF. The apparent "MIC" for *Shig. sonnei* from the viability studies was difficult to determine in CIF but appears to be within the two fold error limit, however that for TSB (0.17mg/ml) is sixteen times lower than the recorded MIC (2.75mg/ml).

These differences in observed antimicrobial activity in different milieu could have one or more explanations: 1) CIF may have a weaker buffering capacity (compared to TSB) against the antimicrobial activity of the G.O - If this is true then the specific effect only relates to certain organisms since this sensitivity was not shown with either *E. coli* or *S. typhimurium* AND 2) *Shig. sonnei* and *L. monocytogenes* react differently to the presence of G.O in CIF than either *E. coli* or *S. typhimurium*. This difference could result from either; A) physiological differences of the cells in the different media OR B) G.O or certain components of G.O react differently in different environments, either with i) cell or ii) media components, such as inhibitory SH-groups. If this statement is correct then it suggests that different components of G.O effect different cells in different ways.

Figure 3.5.1.1b: The Effect Of Various G.O Concentrations On *S. typhimurium* (434) In TSB

Figure 3.5.1.1c: The Effect Of Various G.O Concentrations On *S. typhimurium* (434) In CIF

Serial two-fold dilutions of G.O were prepared in TSB and CIF in tin foil capped boiling tubes to a total volume of 20ml. 40µl of an overnight culture of *S. typhimurium* (434) was used as an inoculum. The tubes were incubated in a water bath at 37°C and the viability of the cells measured with respect to time using the Miles & Misra enumeration technique.

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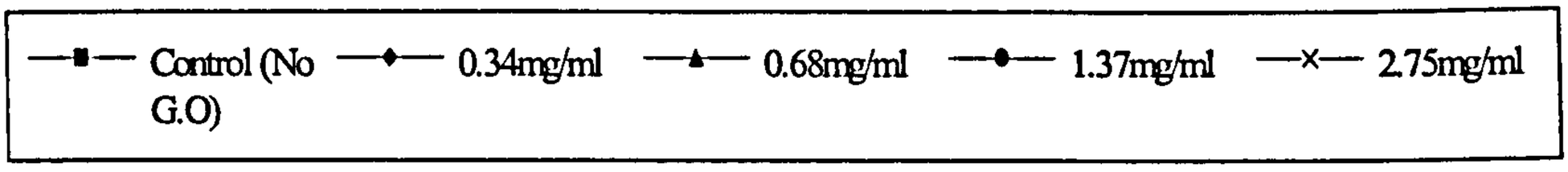

Figure 3.5.1.1 b

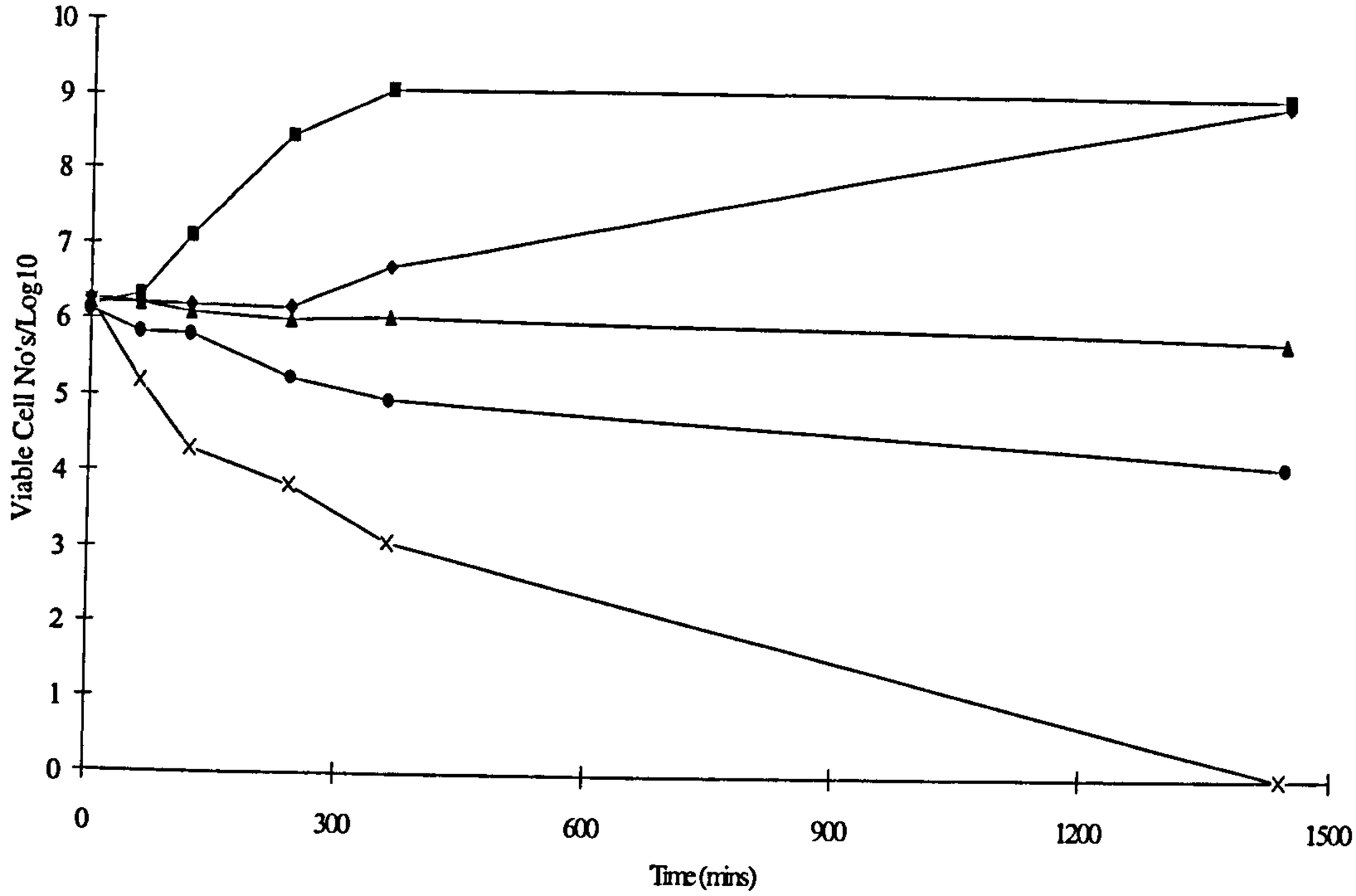

Figure 3.5.1.1 c

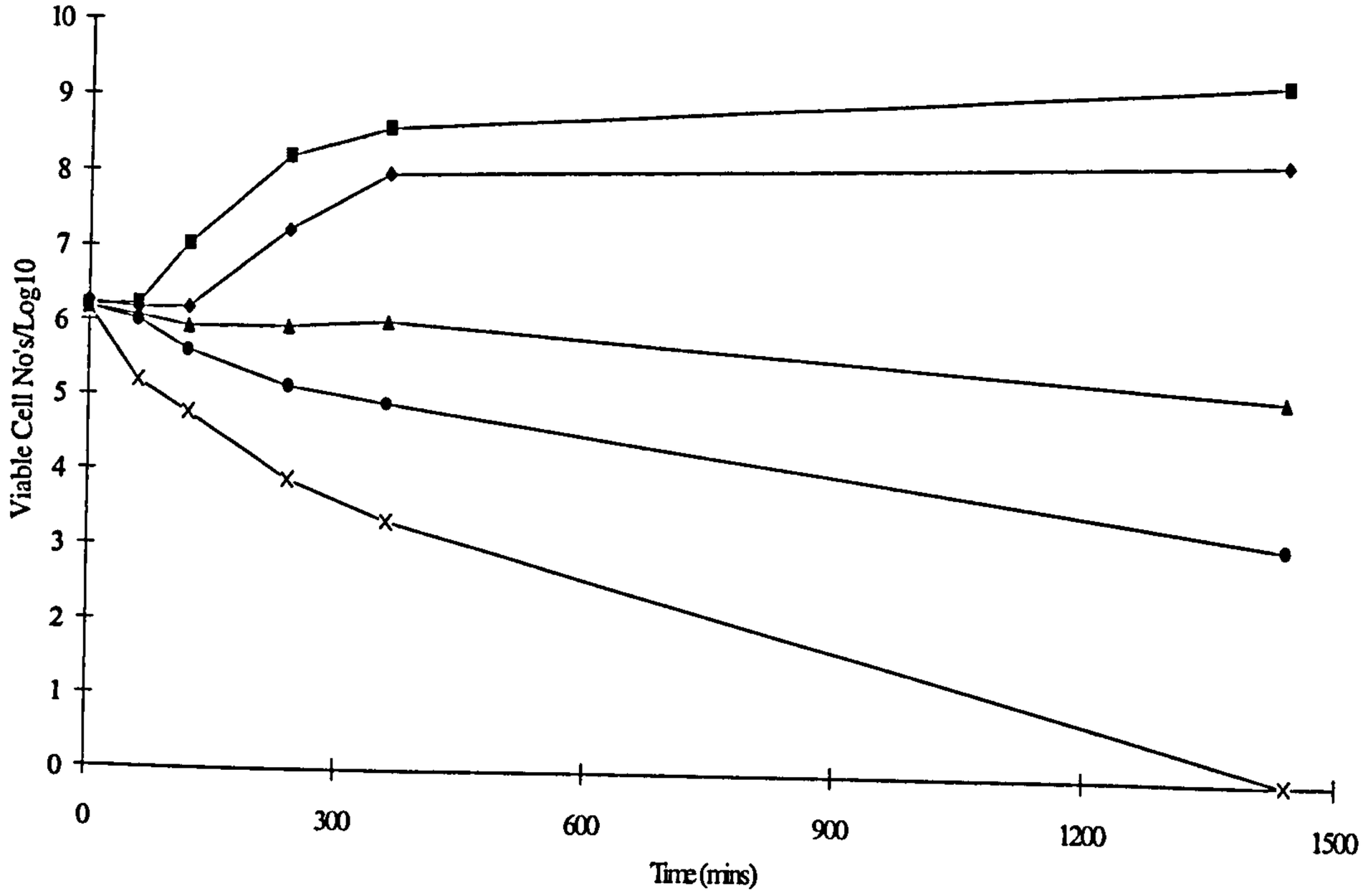

Figure 3.5.1.1d: The Effect Of Various G.O Concentrations On *L. monocytogenes* (433) In TSB

Figure 3.5.1.1e: The Effect Of Various G.O Concentrations On *L. monocytogenes* (433) In CIF

Serial two-fold dilutions of G.O were prepared in TSB and CIF in tin foil capped boiling tubes to a total volume of 20ml. 40µl of an overnight culture of *L. monocytogenes* (433) was used as an inoculum. The tubes were incubated in a water bath at 37°C and the viability of the cells measured with respect to time using the Miles & Misra enumeration technique.

LEGEND:

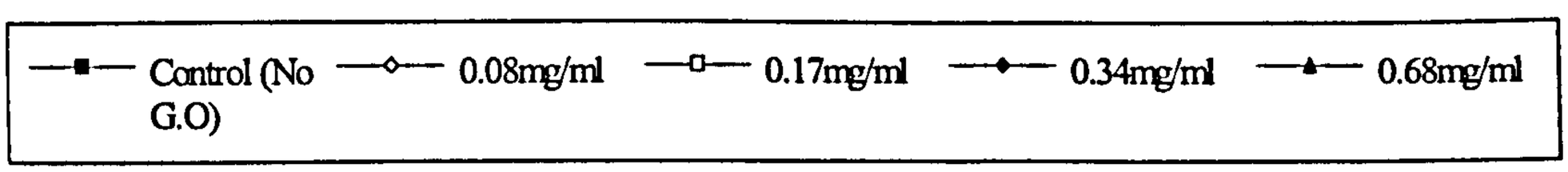

Figure 3.5.1.1 d

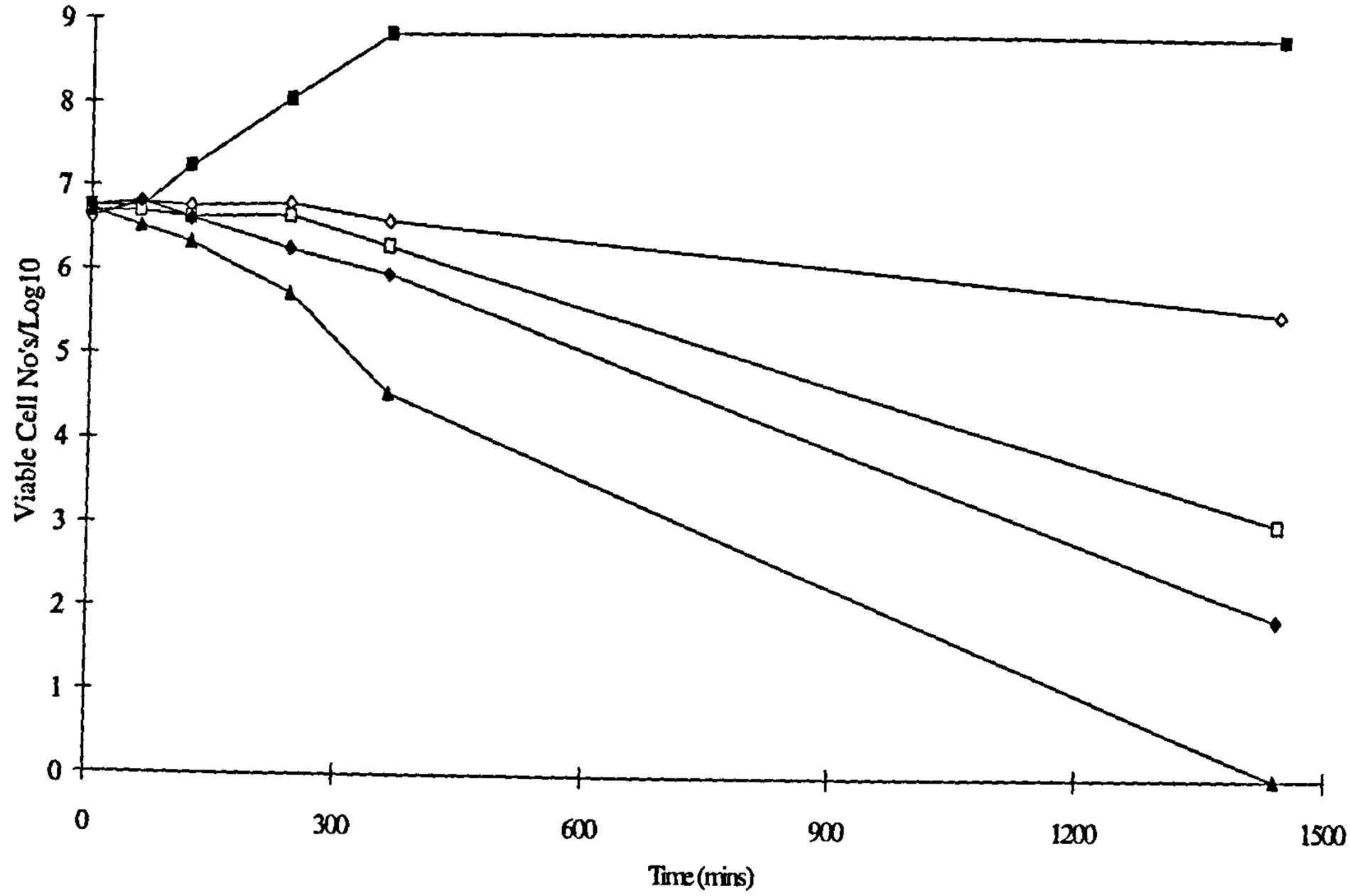

Figure 3.5.1.1 e

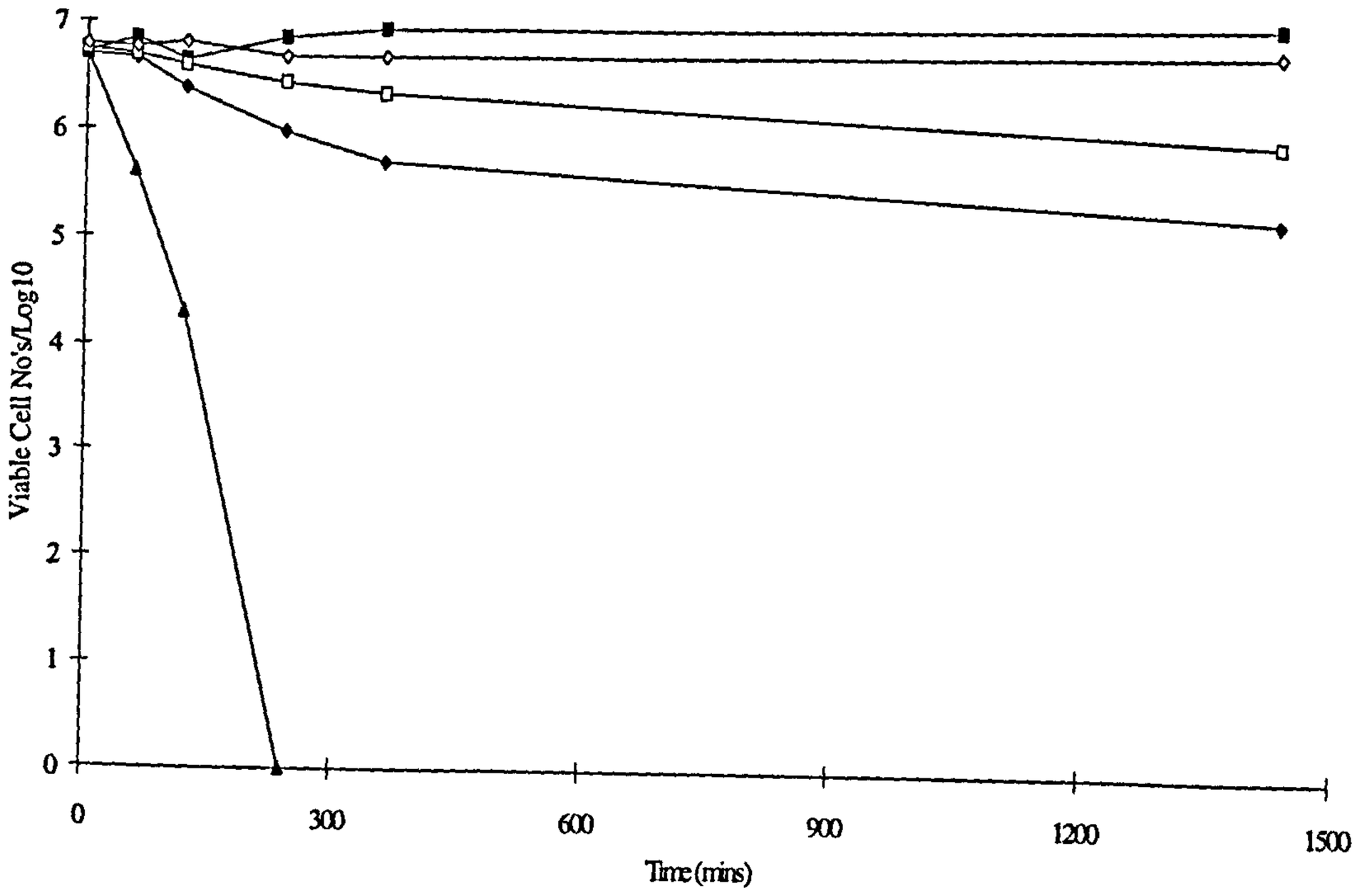

Figure 3.5.1.1f: The Effect Of Various G.O Concentrations On *Shig. sonnei* (426) In TSB

Figure 3.5.1.1g: The Effect Of Various G.O Concentrations On *Shig. sonnei* (426) In CIF

Serial two-fold dilutions of G.O were prepared in TSB and CIF in tin foil capped boiling tubes to a total volume of 20ml. 40µl of an overnight culture of *Shig. sonnei* (426) was used as an inoculum. The tubes were incubated in a water bath at 37°C and the viability of the cells measured with respect to time using the Miles & Misra enumeration technique.

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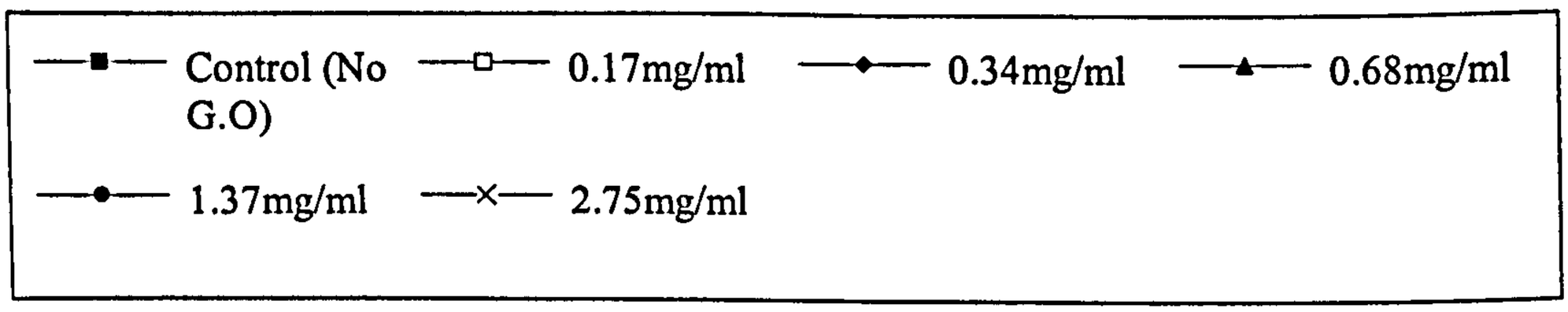


Figure 3.5.1.1 f

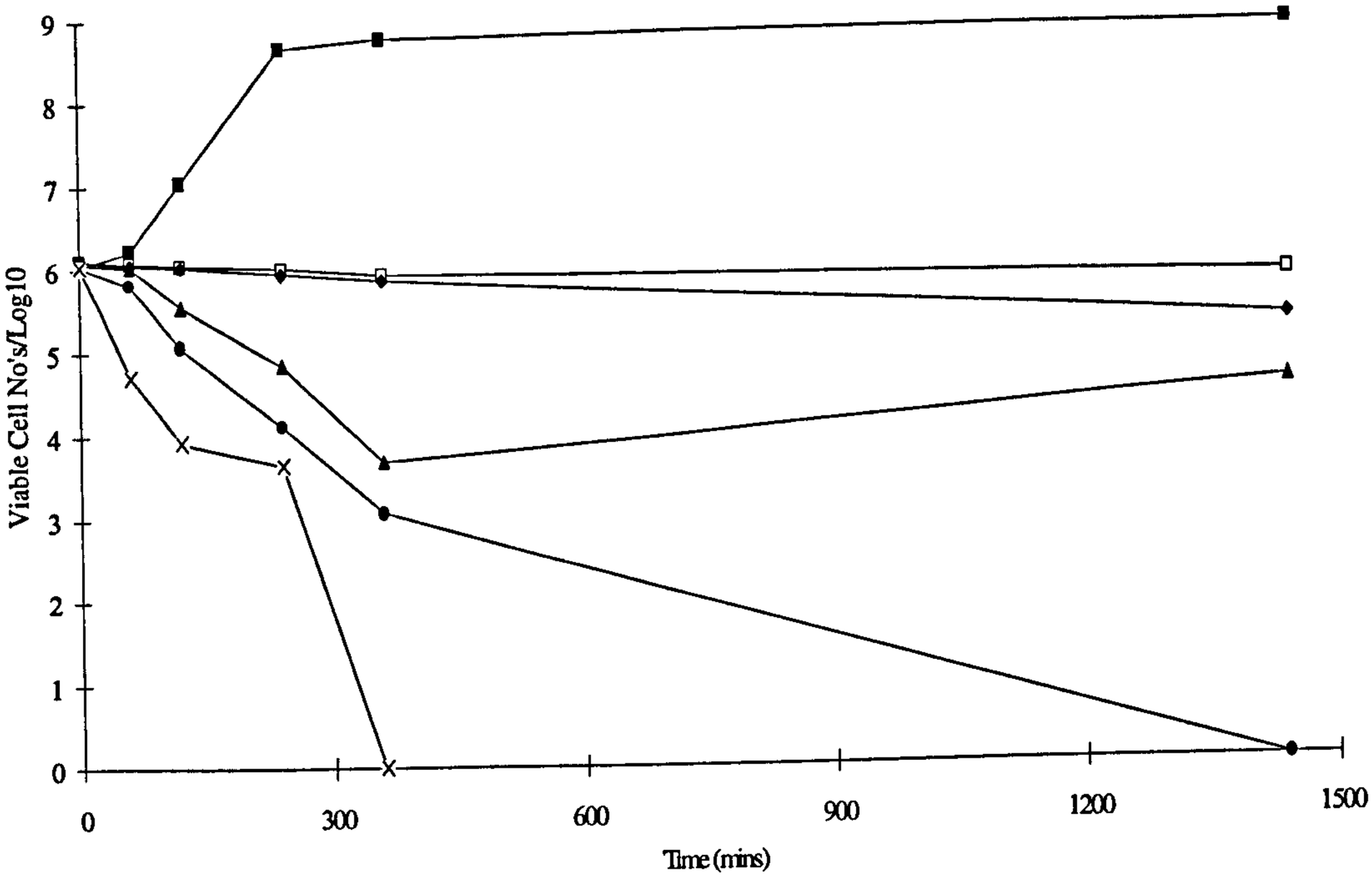
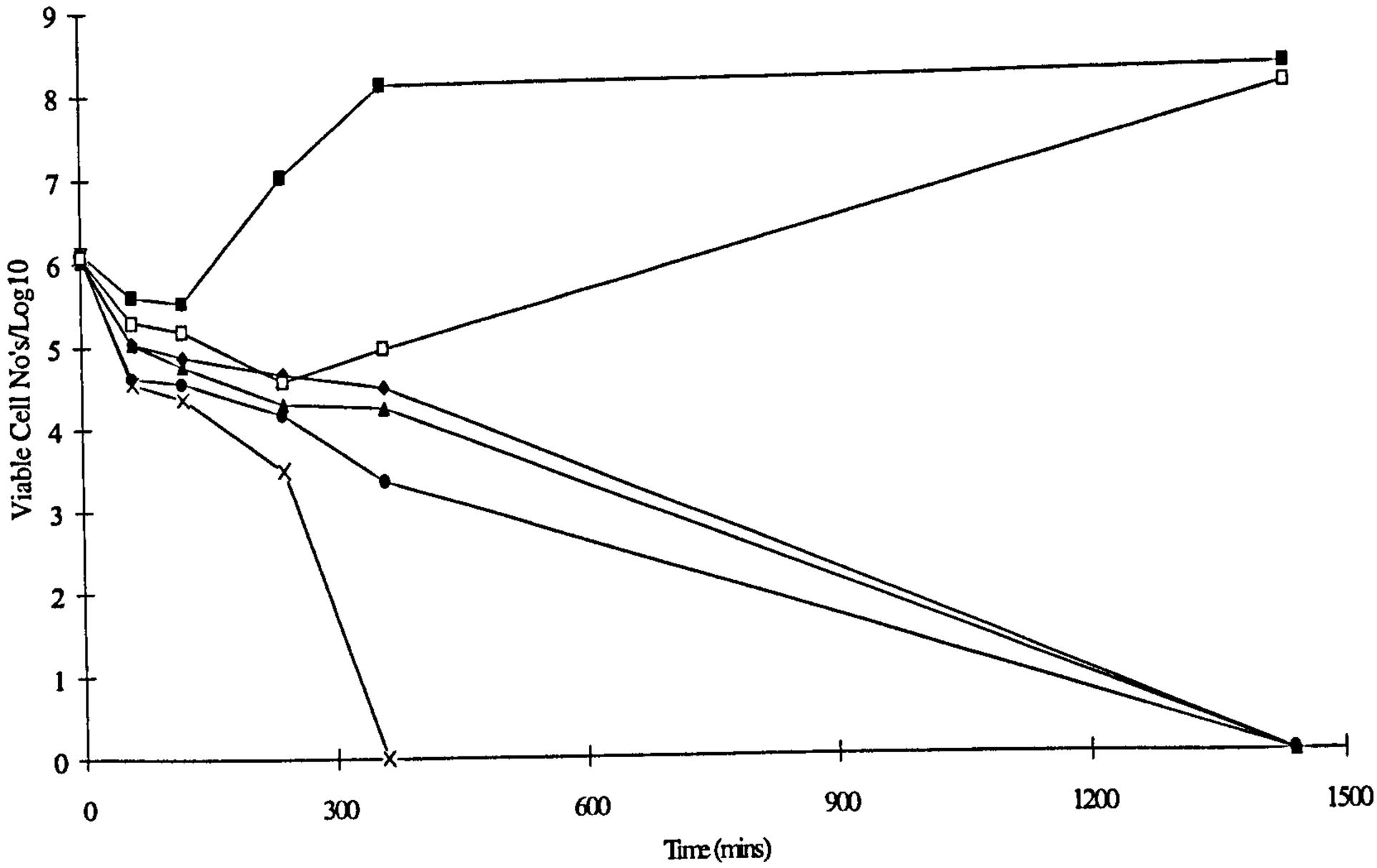


Figure 3.5.1.1 g



The relationship between initial cell death rate and concentration of G.O was then examined as shown in Figures 3.5.1.1h-k.

The data shows that in CIF (as in 3.2f), the rate of loss of cell viability is proportional to the concentration of G.O especially in the range of 0-3mg/ml. It was interesting to note that the ability of G.O to exert antimicrobial activity against all the organisms tested is subject to the environmental conditions, such as media type. The extent of the variation in the effect of G.O in different environments is dependent on the organism under investigation. Thus, with *S. typhimurium* the variation is slight whereas with *L. monocytogenes* and *Shig. sonnei* the antimicrobial activity of G.O is much more accentuated in the CIF environment.

Figure 3.5.1.1h: Comparison Of Initial Cell Death/Growth Rates Of *E. coli* (40) In TSB & CIF

Figure 3.5.1.1i: Comparison Of Initial Cell Death/Growth Rates Of *S. typhimurium* (434) In TSB & CIF

The source of data used for these graphs was derived from the initial cell death/growth rates determined from Figures 3.5.1c, d for *E. coli* (40) and from Figures 3.5.1.1b, c for *S. typhimurium* (434).

LEGEND:

Figure 3.5.1.1 h

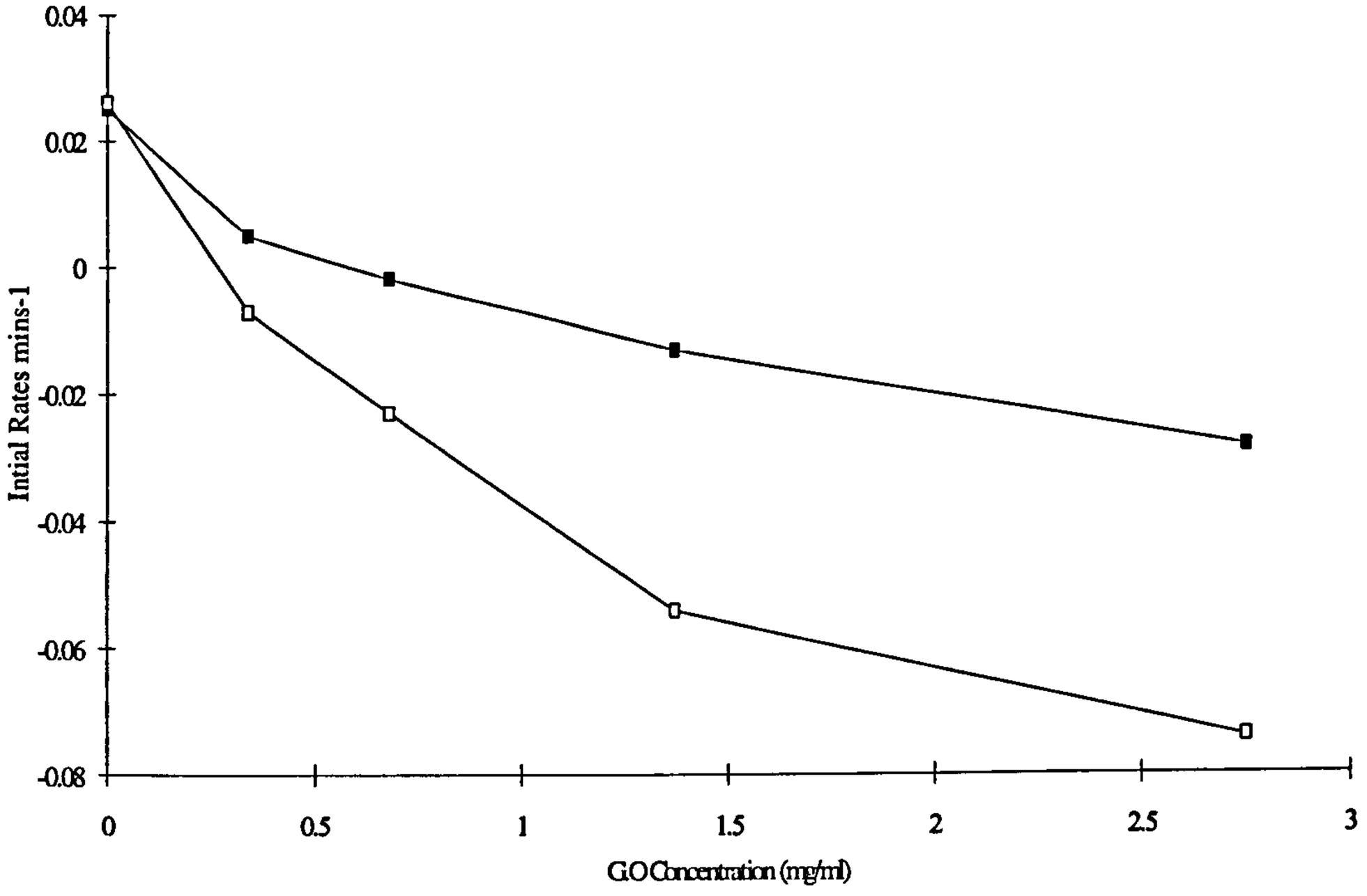


Figure 3.5.1.1 i

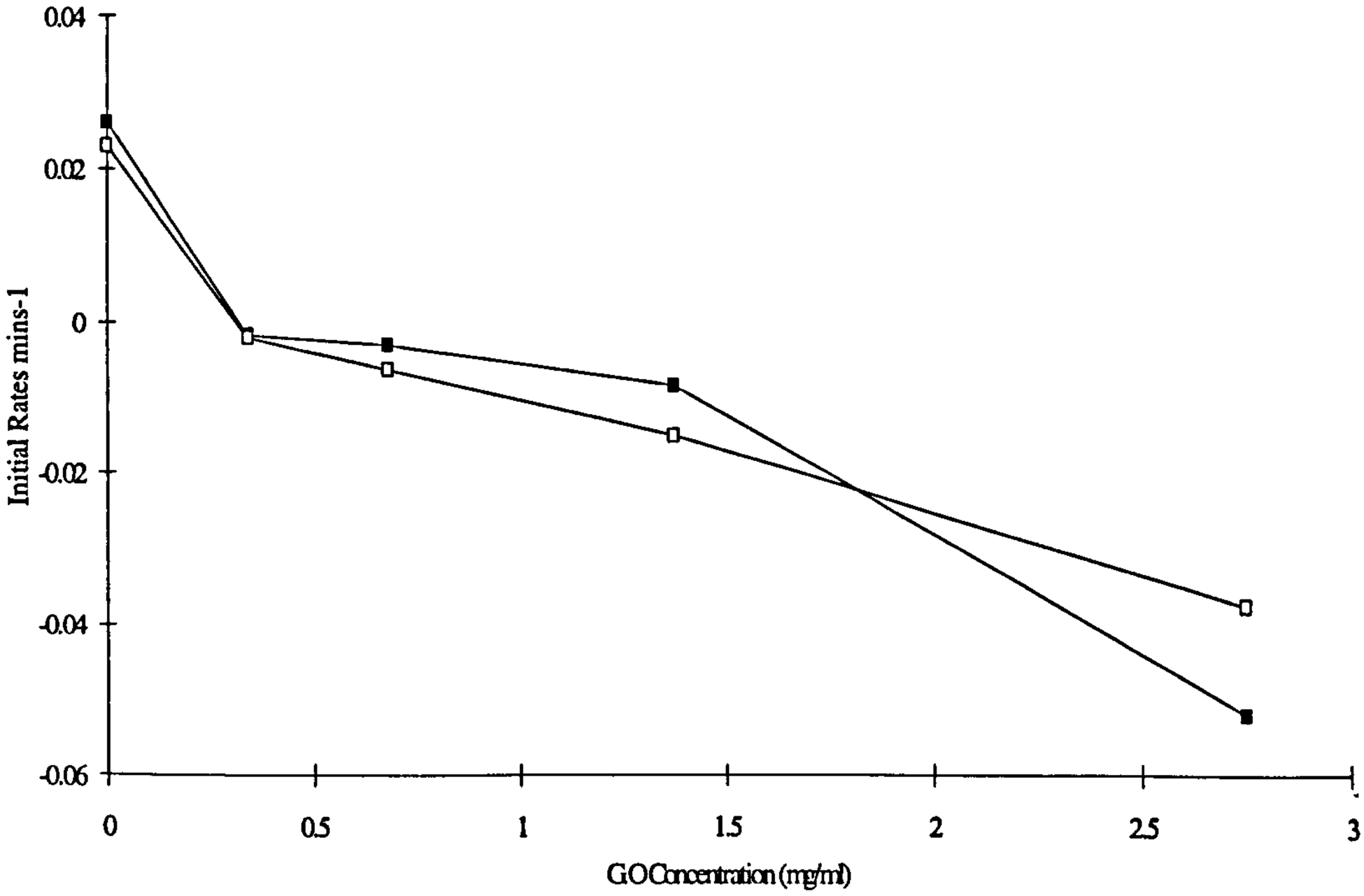


Figure 3.5.1.1j: Comparison Of Initial Cell Death/Growth Rates Of *Shig. sonnei* (426) In TSB & CIF

Figure 3.5.1.1k Comparison Of Initial Cell Death/Growth Rates Of *L. monocytogenes* (433) In TSB & CIF

The source of data for these graphs was derived from the initial cell death/growth rates determined from Figures 3.5.1.1f, g for *Shig. sonnei* (426) and from Figures 3.5.1.1d, e for *L. monocytogenes* (433).

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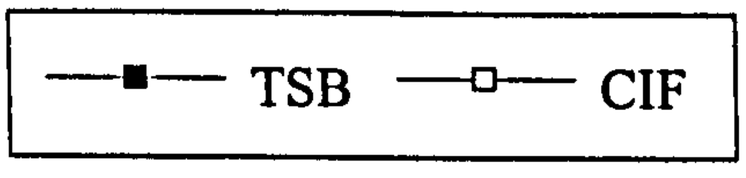


Figure 3.5.1.1 j

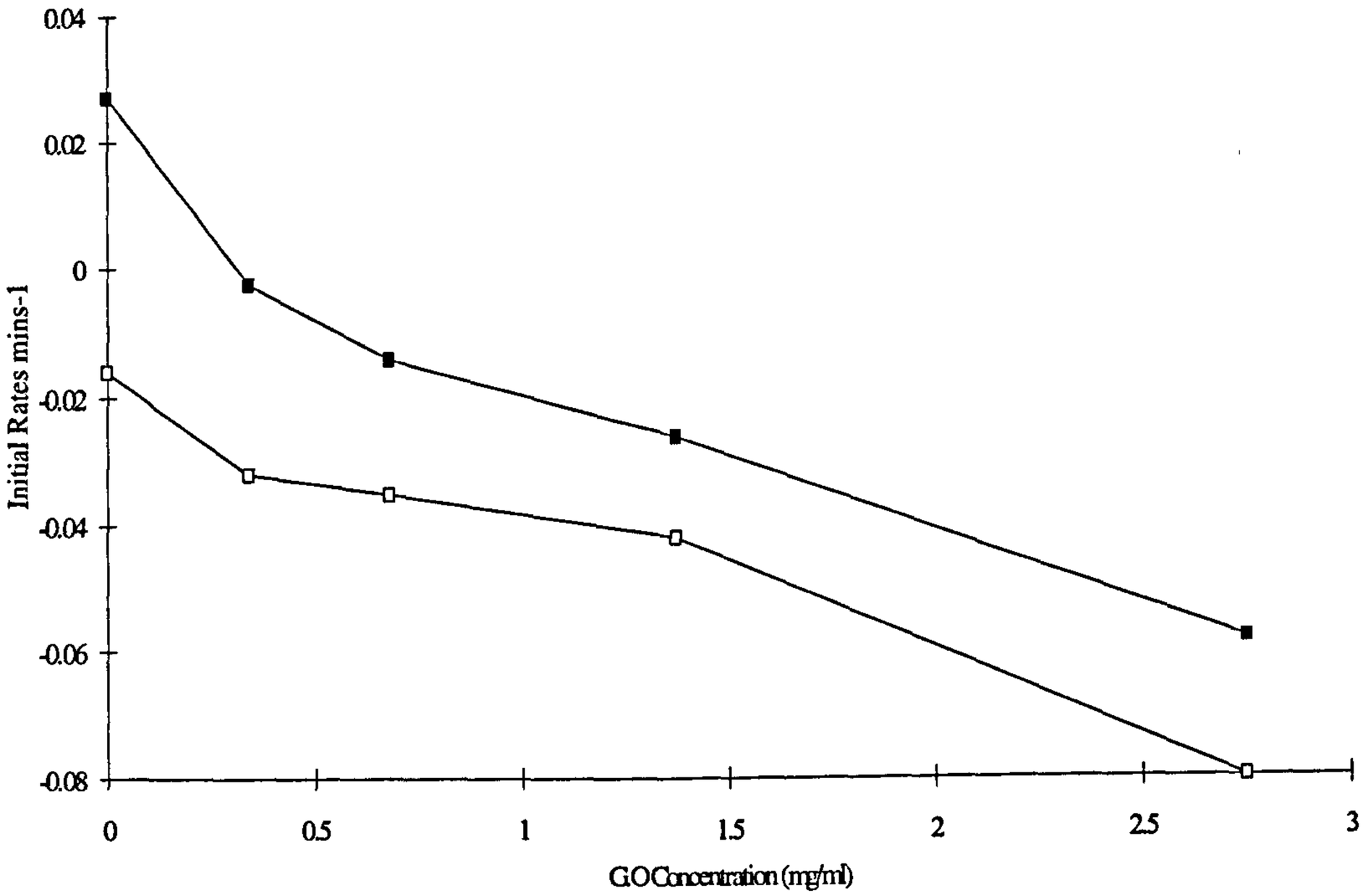
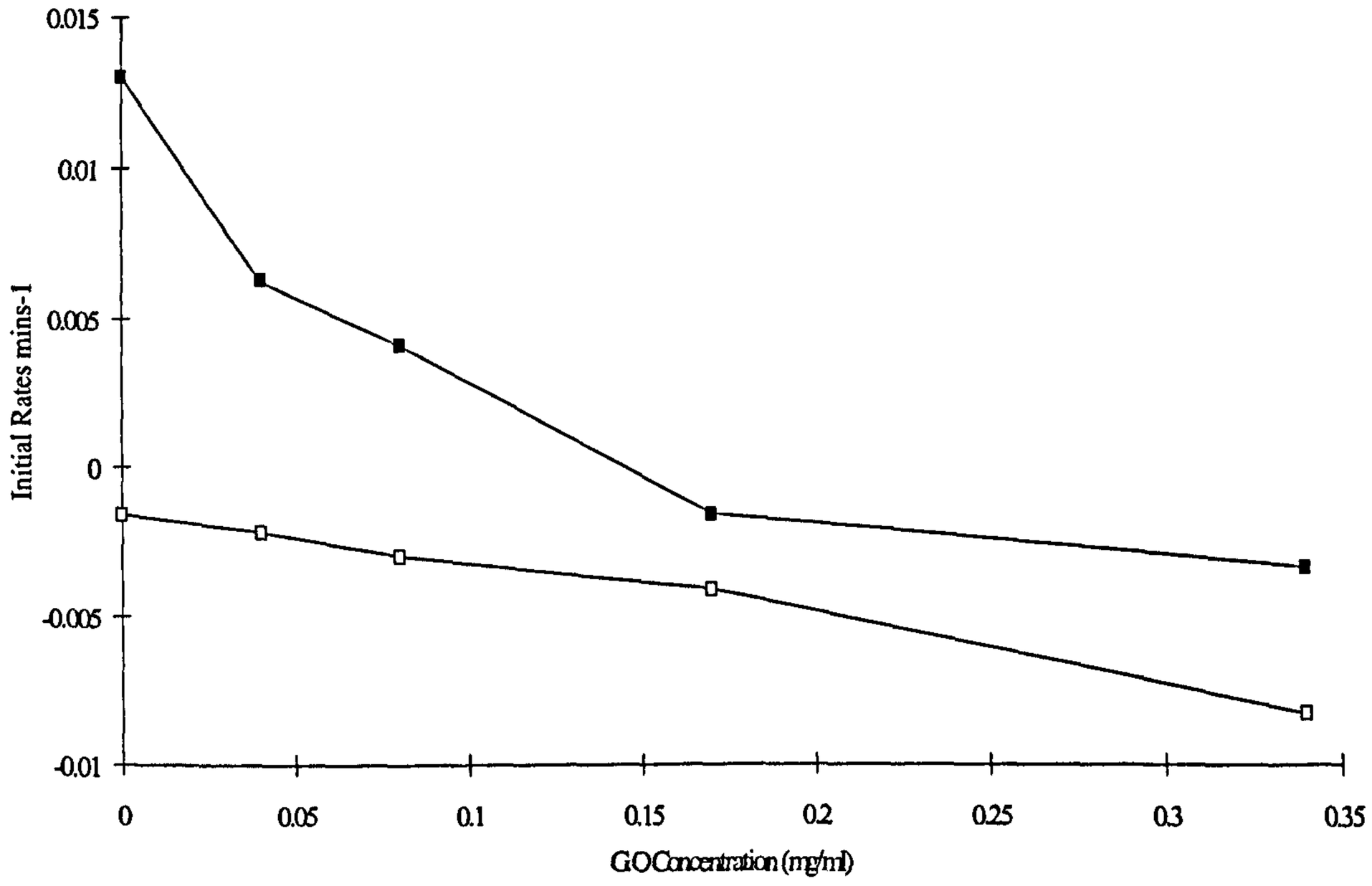


Figure 3.5.1.1 k



3.5.1.2 The Effect Of G.O On A Mixed Culture Of *E. coli* (40) & *L. monocytogenes* (433) In TSB & CIF

Preliminary mixed culture experiments in both TSB and CIF were then performed using *E. coli* (40) and *L. monocytogenes* (433). The aim of this work was to progress towards determining the effect of G.O within a more realistic intestinal environment, that is, in terms of the chemical physiology and microbial composition. As stated in the Introduction (p18), the intestinal tract has a complex microbial ecosystem. The first approach to investigating the impact of microbial interaction upon the antimicrobial activity of G.O involved the use of two species. *E. coli* and *L. monocytogenes* were chosen as both display differences in sensitivity towards G.O, also *L. monocytogenes* could be differentially distinguished from *E. coli* on LSA (Listeria Selective Agar). The results are represented in Figures 3.5.1.2a, b.

Figure 3.5.1.2a: The Effect Of G.O On A Mixed Culture Of *E. coli* & *L. monocytogenes* In TSB

Serial two-fold dilutions of G.O in TSB were prepared in tin foil capped boiling tubes to a total volume of 20ml. To each tube 20µl of an overnight culture of both organisms was added as an inoculum. The tubes were incubated at 37°C in a water bath. Viable counts were performed with respect to time using either the spread plate or Miles & Misra techniques.

Note: Assuming the *L. monocytogenes* colonies on TSA are equal to those on LSA, the number of *E. coli* cells was determined by subtracting the numbers of *L. monocytogenes* cells on the LSA plates from the total counts of bacteria obtained on the TSA plates.

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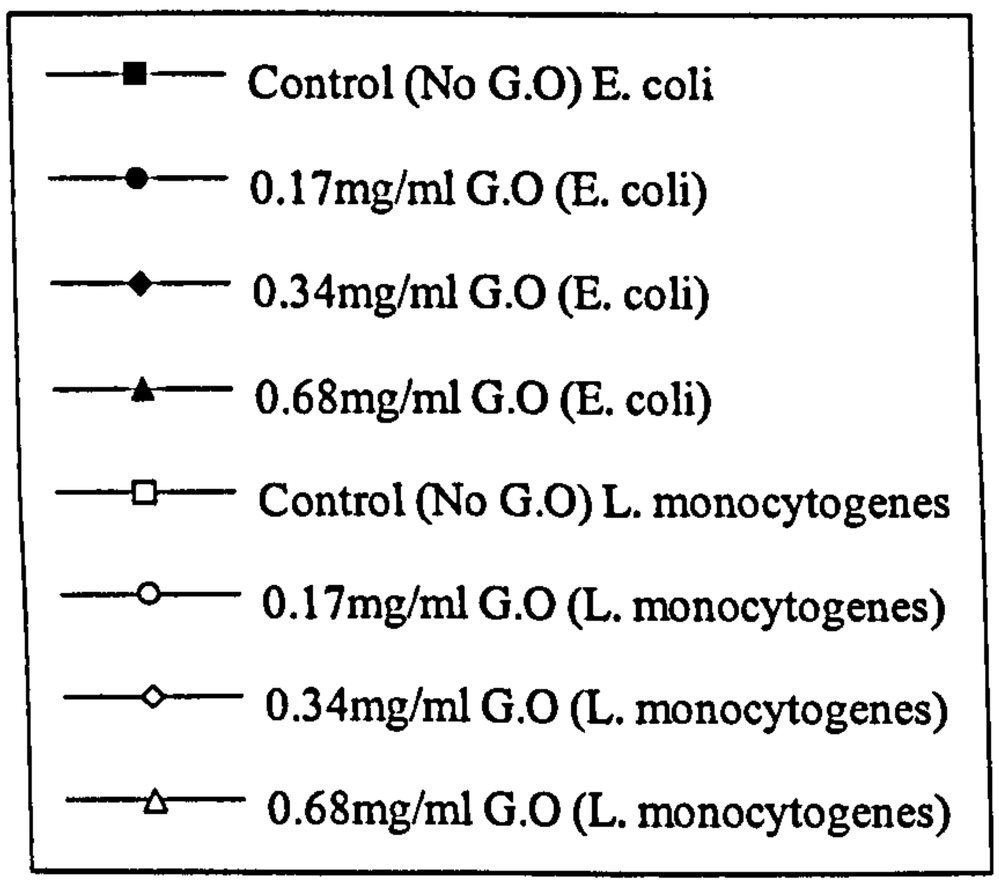
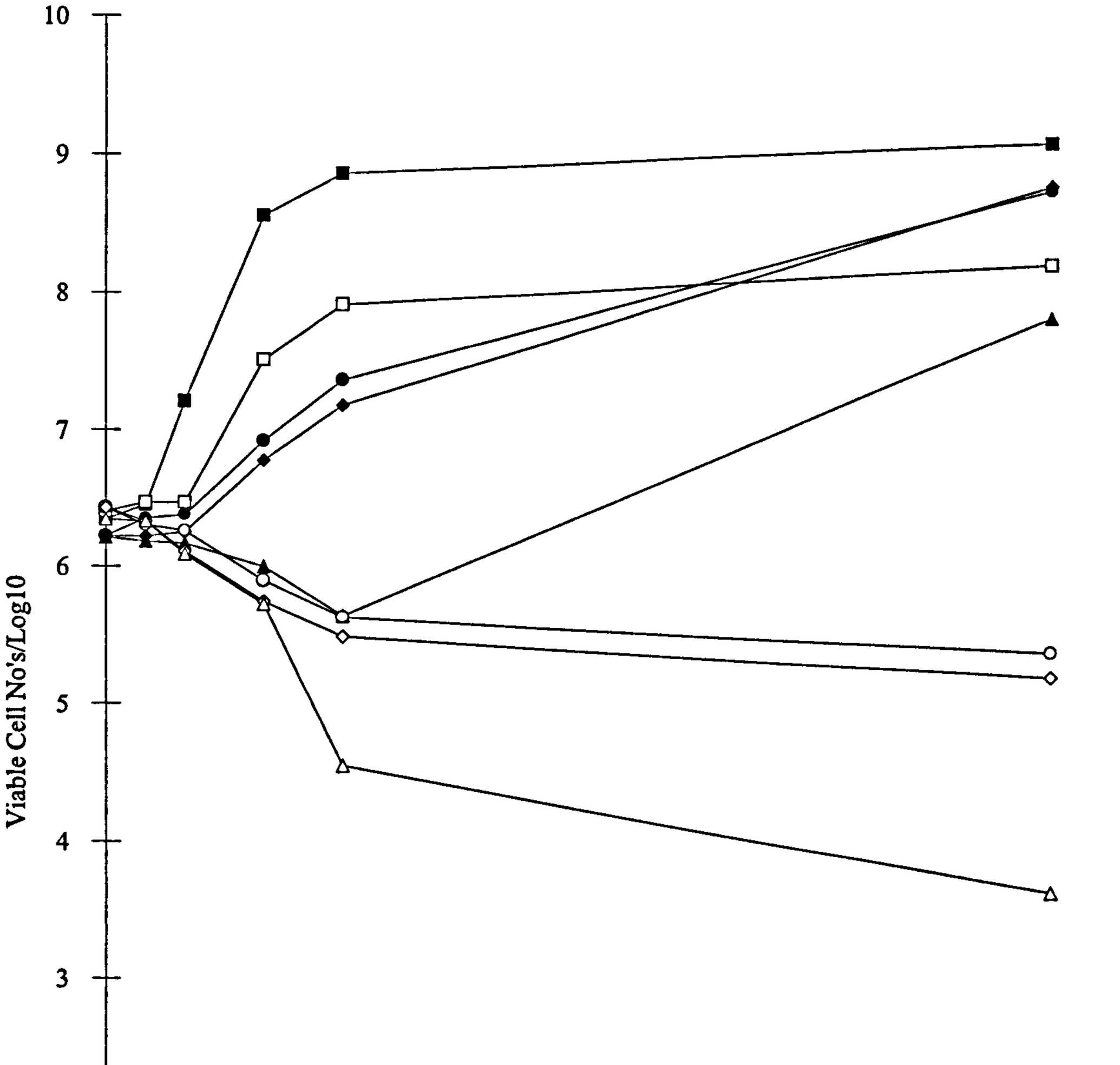


Figure 3.5.1.2 a



Comparison of these mixed culture results with those obtained in the pure culture studies for both *E. coli* and *L. monocytogenes* in TSB (Figures 3.5.1c and 3.5.1.1d), indicated a number of differences; 1) No extended 'lag' phases were observed in the mixed culture studies. 2) At the highest G.O concentration (0.68mg/ml), *E. coli* cells overcome an initial decline in population in the mixed culture which was not observed in the pure culture studies. In the mixed culture study, the *L. monocytogenes* cells show a significant decline in cell population size but not to the same extent as in the pure culture study where complete kill was observed for the same G.O concentration of 0.68mg/ml. These results could reflect a G.O "neutralising" effect resulting from the increased microbial biomass present as compared to the situation in pure cultures. 3) A decrease in the final population size of both organisms in the control was observed in the mixed culture studies compared to the pure culture studies. This could be explained by competition for nutrients between both organisms. 4) As in pure culture studies *E. coli* recovered from G.O inhibition whereas *L. monocytogenes* continued to decline, although unlike the situation in pure culture at the concentration of 0.68mg/ml this decline did not end in complete kill of the organism.

Initial cell population growth rates of both organisms in the absence of G.O were similar to those obtained in pure culture. Data obtained from the effect of G.O on pure cultures of *E. coli* and *L. monocytogenes* in TSB (Figures 3.5.1.1h and k) confirmed that the *L. monocytogenes* cells were more sensitive to G.O than *E. coli* cells.

Figure 3.5.1.2b: The Effect Of G.O On A Mixed Culture Of *E. coli* & *L. monocytogenes* In CIF

Serial two-fold dilutions of G.O in CIF were prepared in tin foil capped boiling tubes to a total volume of 20ml. To each tube 20µl of both organisms was added as an inoculum. The tubes were incubated at 37°C in a water bath. Viable counts were performed with respect to time using either the spread plate or Miles & Misra techniques.

Note: Assuming the *L. monocytogenes* colonies on TSA are equal to those on LSA, the number of *E. coli* cells was determined by subtracting the numbers of *L. monocytogenes* cells on the LSA plates from the total counts of bacteria obtained on the TSA plates.

LEGEND:

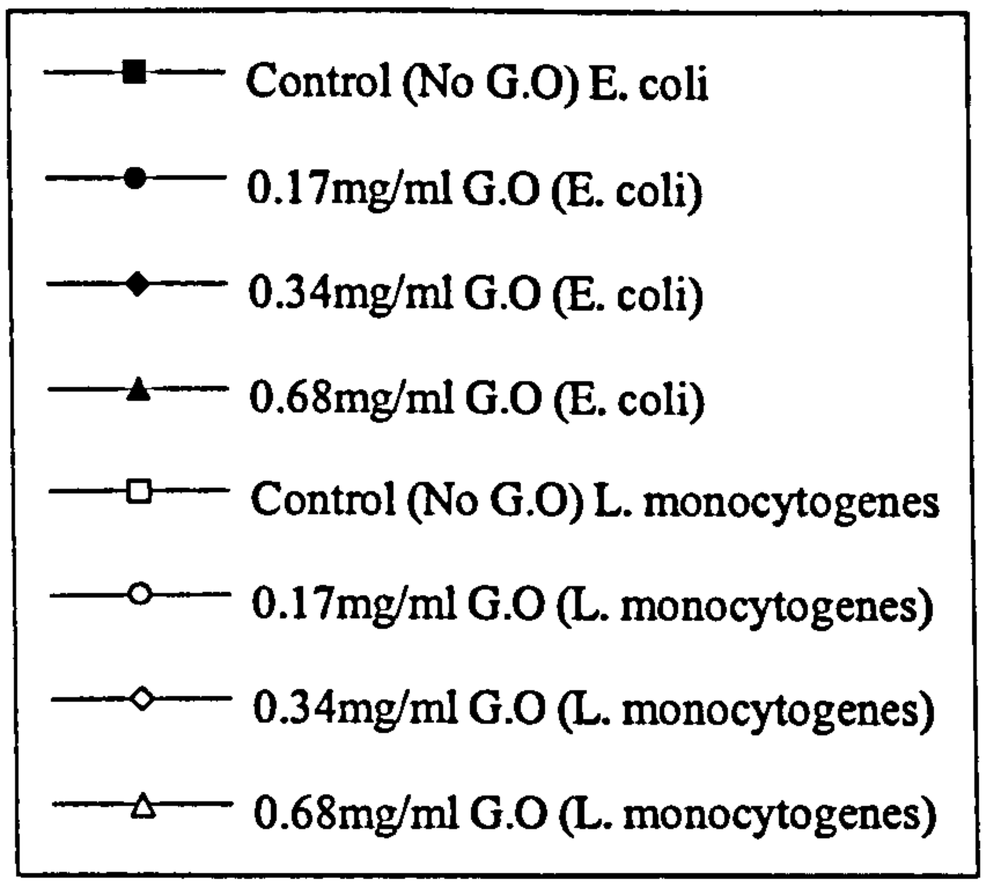
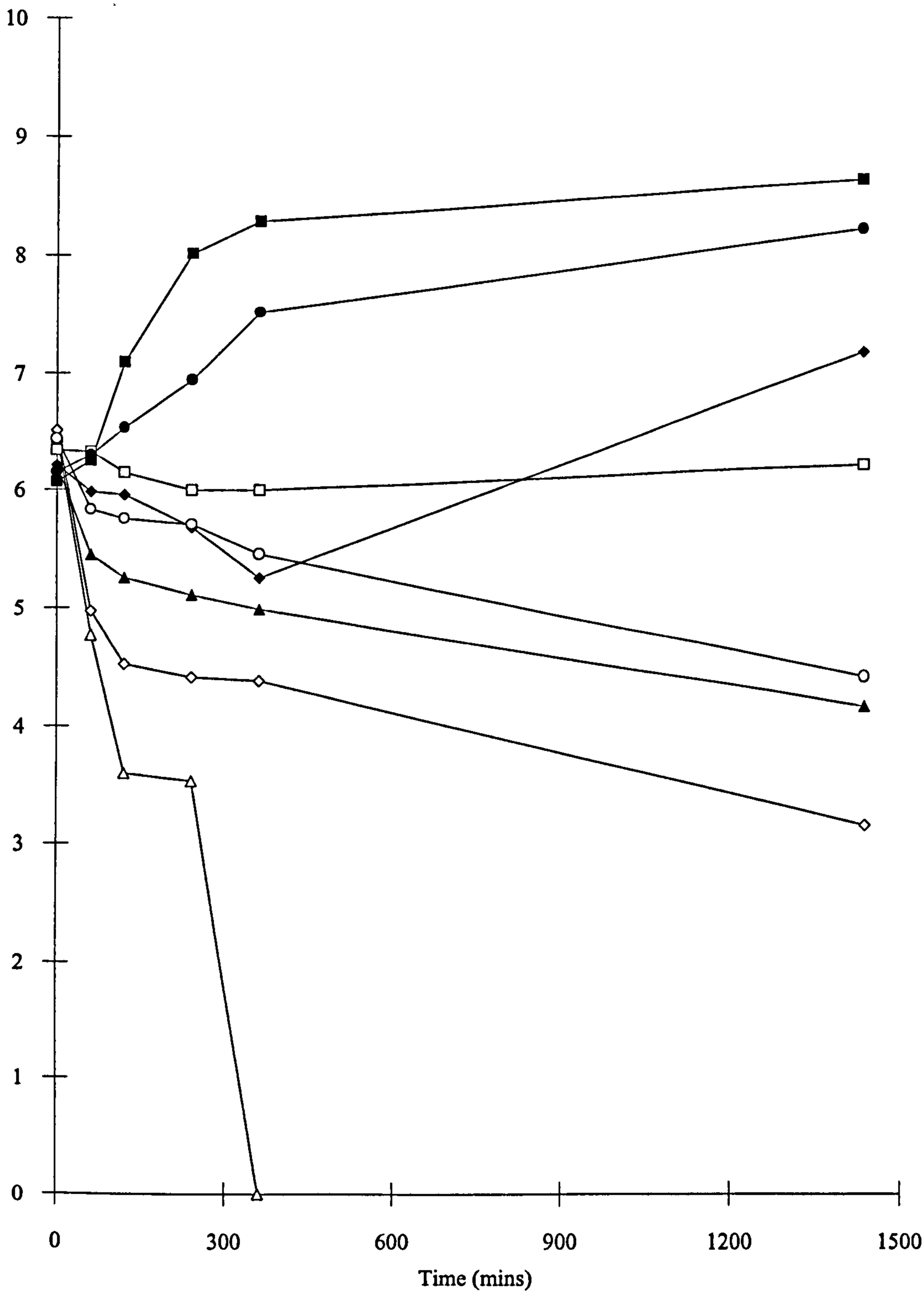


Figure 3.5.1.2 b



Similar results were obtained in CIF to those in TSB, however both organisms appear to be more sensitive to G.O in CIF. This was also found to be the case when comparing pure culture results in CIF and TSB (Figures 3.5.1.1h and k). In CIF complete kill of *L. monocytogenes* was obtained using 0.68mg/ml G.O, which was accompanied by a significant decrease in the *E. coli* population.

Comparison of the initial cell death rates (See Figure 3.5.1.2c, d) for *E. coli* in mixed culture to those in pure culture (Figure 3.5.1c, d) were similar. However those of *L. monocytogenes* were much faster in the mixed culture than in pure culture.

Figure 3.5.1.2c: Comparison Of Initial Cell Death/Growth Rates For *E. coli* (40) In Mixed Culture To That Of Pure Culture In CIF

Figure 3.5.1.2d: Comparison Of Initial Cell Death/Growth Rates For *L. monocytogenes* (433) In Mixed Culture To That Of Pure Culture In CIF

The source of data for these graphs was derived from the initial cell death/growth rates determined from Figures 3.5.1d, 3.5.1.1e with Figures 3.5.1.2a, b and plotted as the means of 3 replications.

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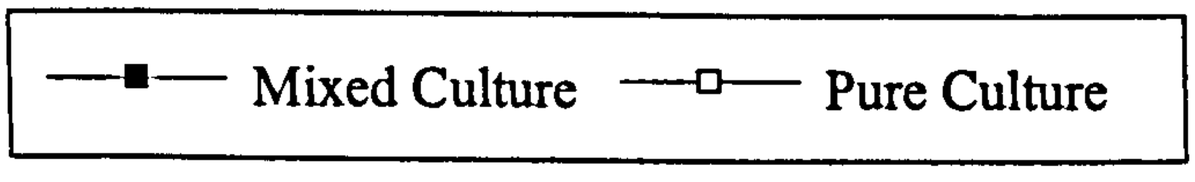


Figure 3.5.1.2c

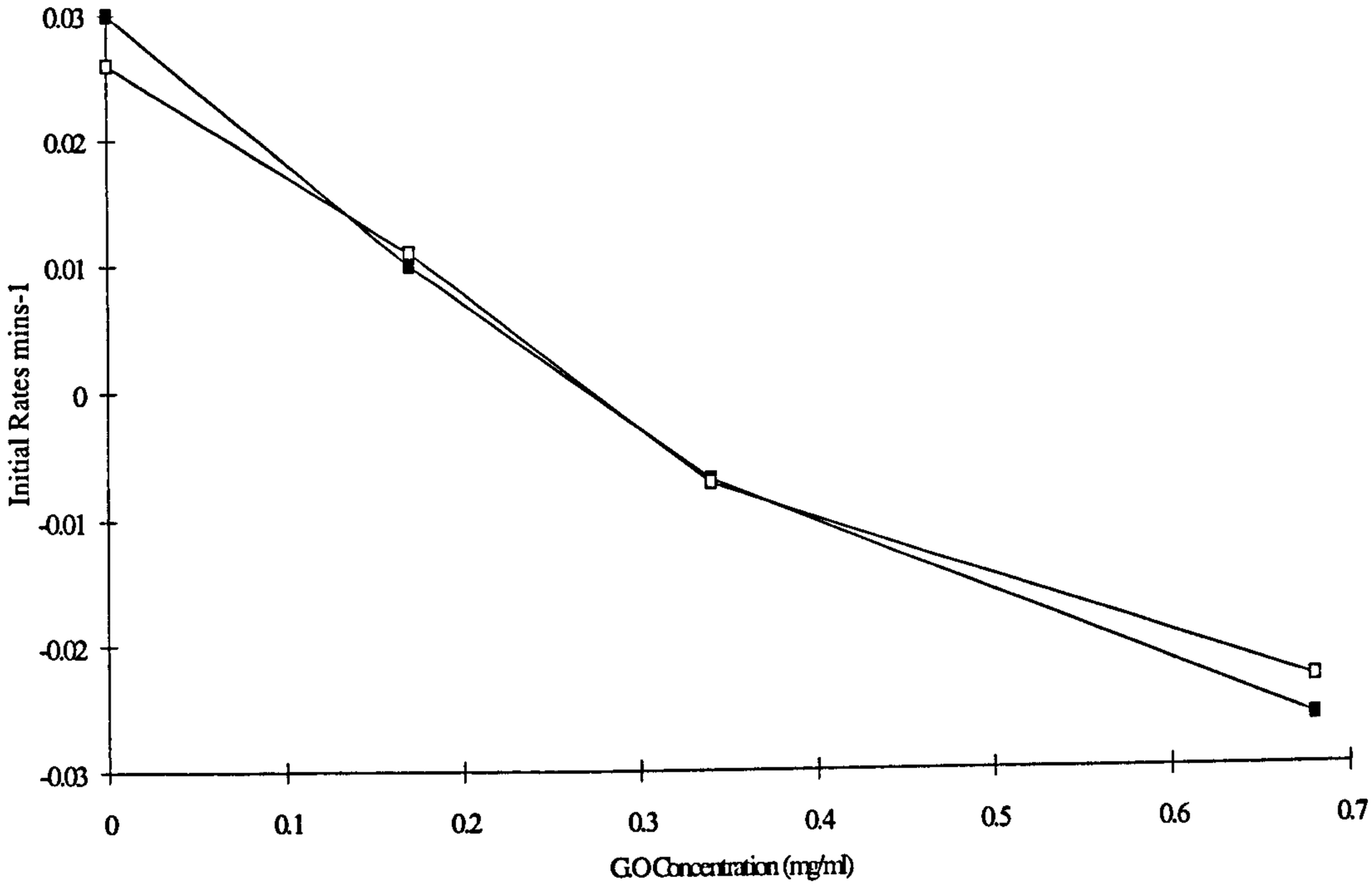
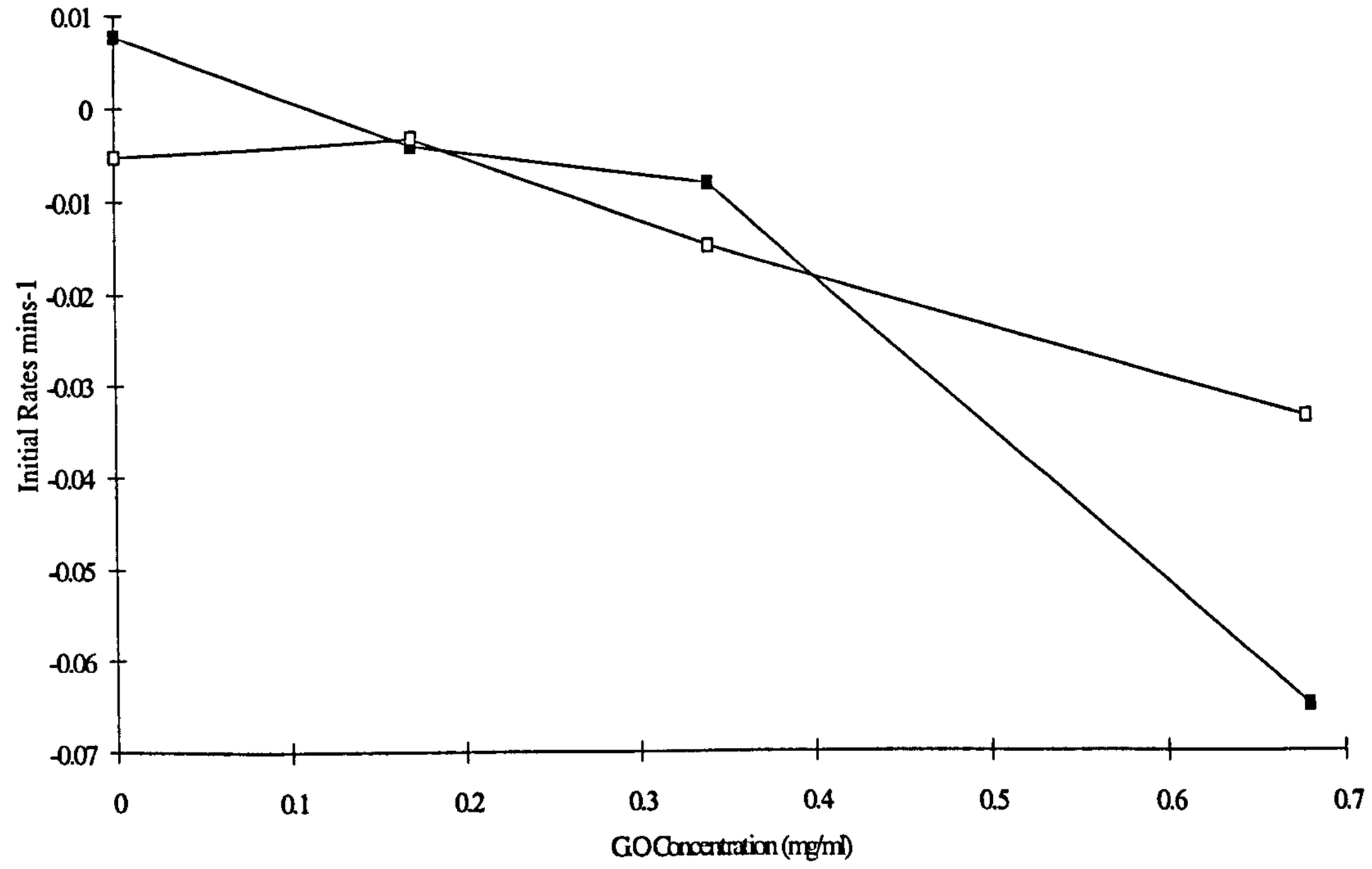


Figure 3.5.1.2d



3.5.2 The Effect Of G.O On Pure & Mixed Cultures Of *L. monocytogenes* (433) And *L. acidophilus* NCFM (Gilliland Strain) In MRS Broth

In order to explore the antimicrobial activity of G.O upon the natural intestinal flora and intestinal pathogens further within a variety of media, it was decided to continue the mixed culture work with *L. acidophilus* (an organism believed to play an important role in the maintenance of a healthy digestive system, Macfarlane & Cummings, 1992) as well as with *L. monocytogenes* (a known food-borne pathogen).

Initial pure culture viability studies of *L. acidophilus* and *L. monocytogenes* were performed in MRS broth using identical G.O concentrations (on repeated occasions). MRS broth was chosen instead of TSB as *L. acidophilus* does not grow in TSB. The typical results are presented in Figures 3.5.2a, b.

Figure 3.5.2a: The Effect Of Various G.O Concentrations On *L. acidophilus* NCFM (Gilliland) In MRS Broth

Figure 3.5.2b: The Effect Of Various G.O Concentrations On *L. monocytogenes* (433) In MRS Broth

Separate serial two-fold dilutions of G.O were prepared in MRS broth in tin foil capped boiling tubes, to a total volume of 20ml. 20µl of overnight cultures of *L. acidophilus* NCFM (Gilliland) and *L. monocytogenes* (433) were used added as an inoculum. The tubes were incubated in a water bath at 37°C and the viability of the cells measured with respect to time using the Miles & Misra enumeration technique.

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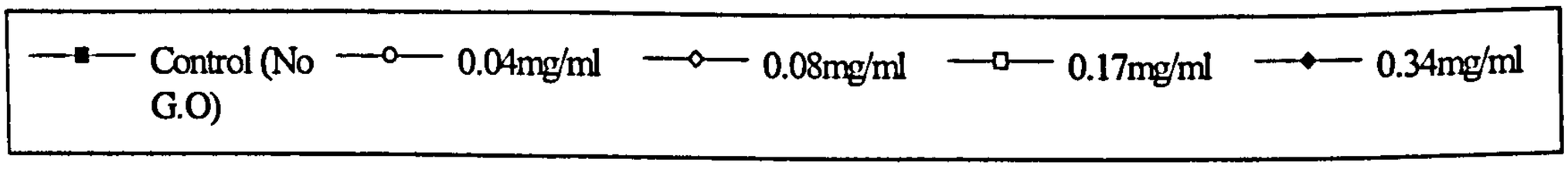

Figure 3.5.2a

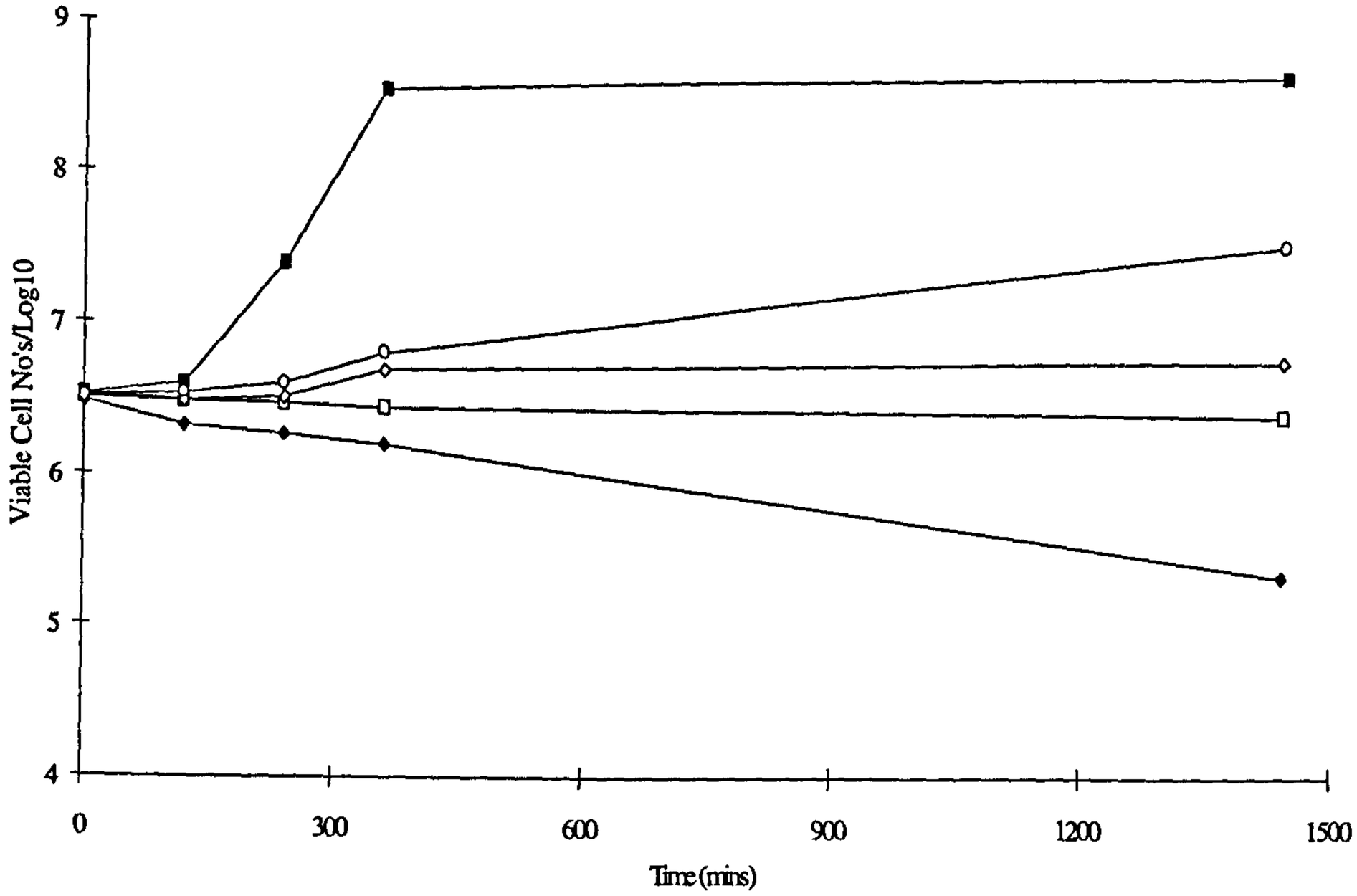

Figure 3.5.2b

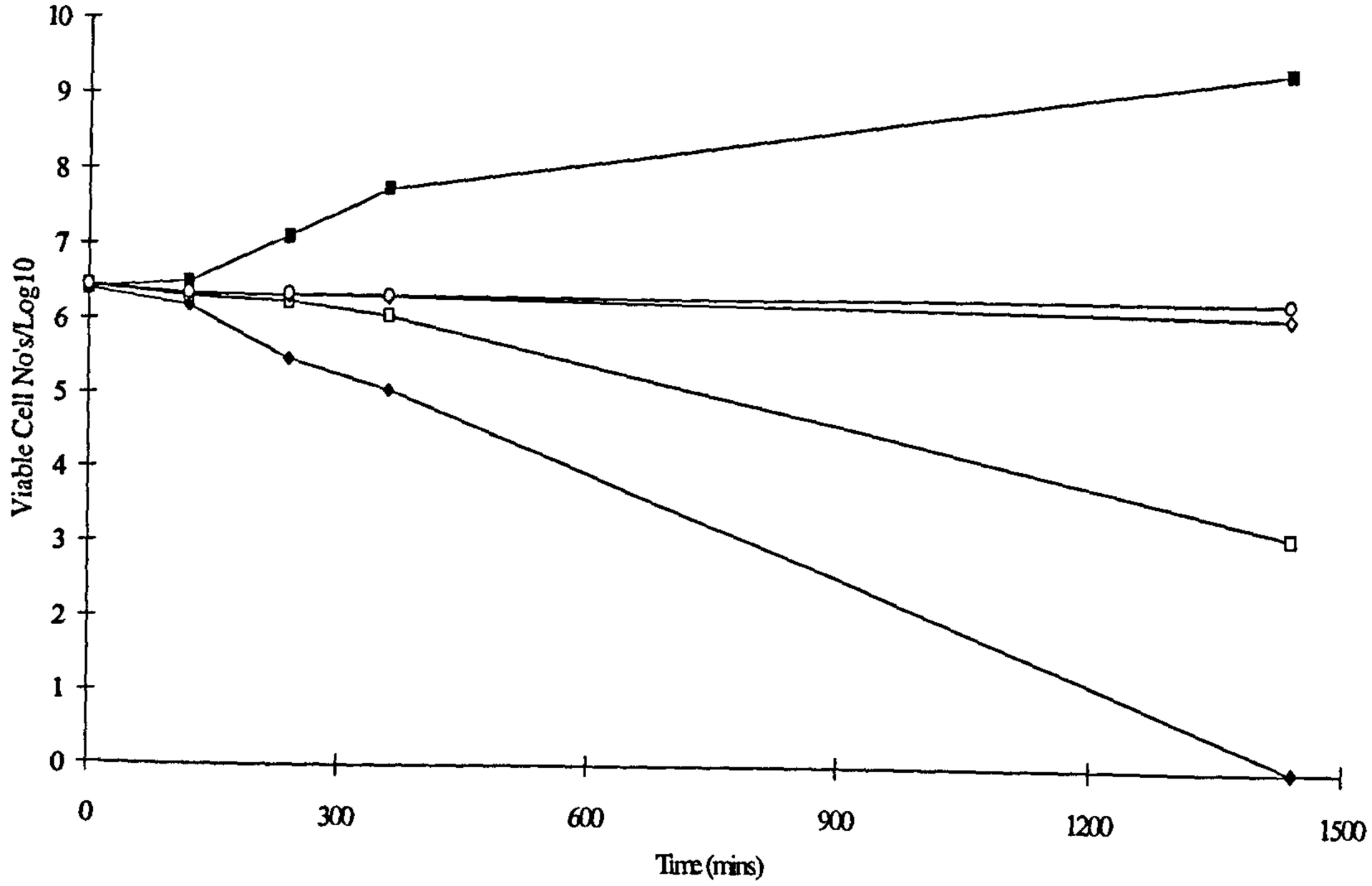

Figure 3.5.2a (*L. acidophilus*) shows that in the absence of G.O (control) the cells grow and proliferate, whereas in the presence of G.O, inhibition of growth or cell death was observed, the rate of which was concentration dependent. Closer observation indicates that in the presence of G.O (0.04 and 0.08mg/ml) a 'lag' phase occurs before *L. acidophilus* cells reproduce, the length of which appears to be concentration dependent. Complete inhibition of growth without any apparent loss of cell viability occurred at 0.17mg/ml. At the highest concentration (0.34mg/ml) neither lag nor plateau stages were seen progressive bacterial death commenced immediately, but over the 24 hour period only an approximate 10-fold decline in *L. acidophilus* cells occurred.

Figure 3.5.2b (*L. monocytogenes*) indicates a similar general pattern in results to those obtained with *L. acidophilus*. Thus in the absence of G.O, *L. monocytogenes* proliferated in the MRS broth, but at a much slower growth rate than that of *L. acidophilus*, whilst in the presence of G.O distinct differences were observed. At the lowest concentrations (0.04 and 0.08mg/ml) slight inhibition of growth was observed, whereas at 0.17mg/ml a 'lag' is observed before substantial cell death commences. At the highest concentration (0.34mg/ml) a short period of slight population decline is followed by a steep progressive decline resulting in complete death of *L. monocytogenes* by 24 hours.

These results suggest, that G.O treatment of a simple mixed bacterial culture of *L. acidophilus* together with the garlic sensitive pathogenic *L. monocytogenes*, would lead to the pathogenic bacterium being eliminated allowing the growth of the natural intestinal bacterium. This hypothesis was further tested by repeating the experiment precisely as previously performed in Figures 3.5.2a, b but as a mixed co-culture. The results are presented in Figures 3.5.2c, d.

Figure 3.5.2c: The Effect Of Various G.O Concentrations On A Mixed Culture Of *L. acidophilus* NCFM (Gilliland) And *L. monocytogenes* (433) In MRS Broth

Figure 3.5.2d: Expanded View Over The First 360 Minutes

Serial two-fold dilutions of G.O were prepared in MRS broth in tin foil capped boiling tubes, to a total volume of 20ml. 20µl of an overnight culture of *L. acidophilus* NCFM (Gilliland) and *L. monocytogenes* (433) were used as the inoculum. The tubes were incubated in a water bath at 37°C and the viability of the cells measured with respect to time using the Miles & Misra enumeration technique.

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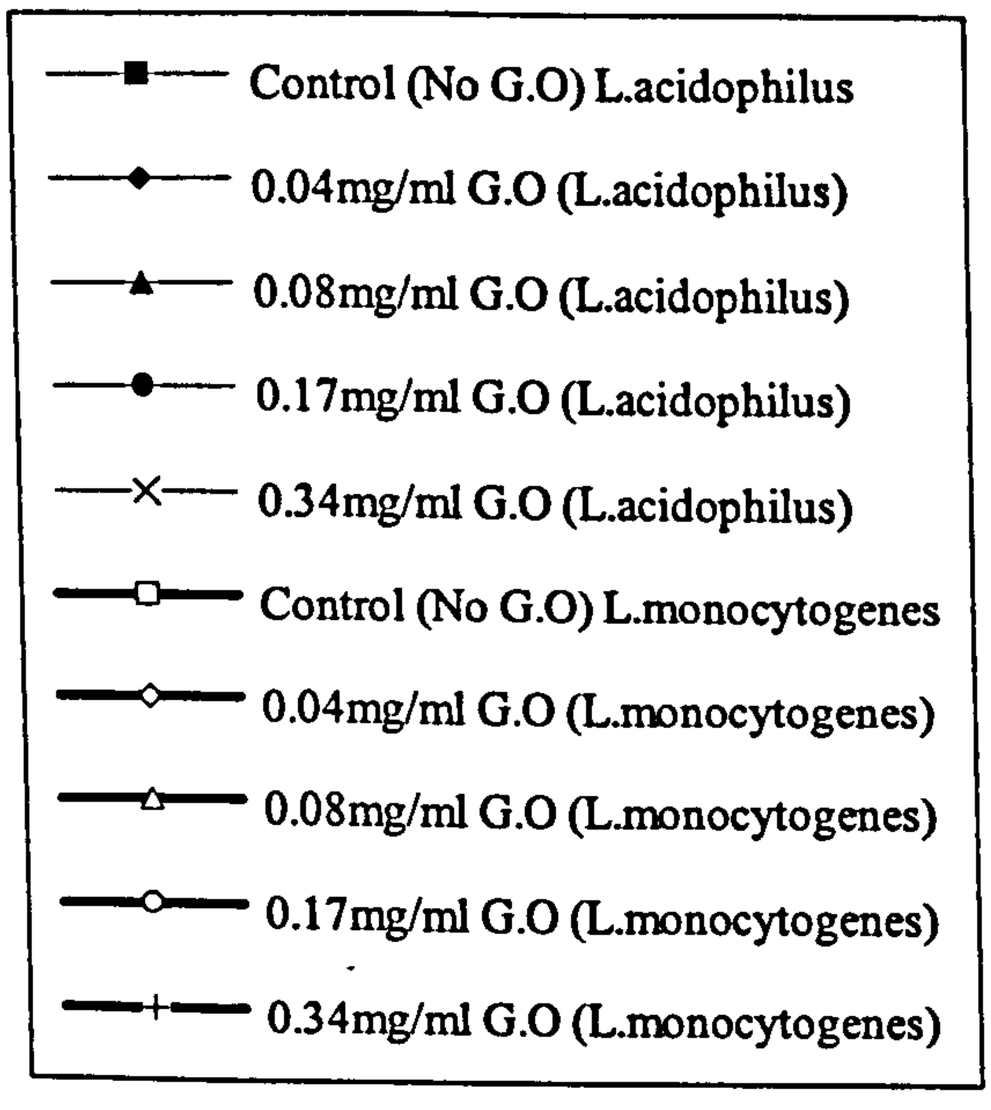


Figure 3.5.2c

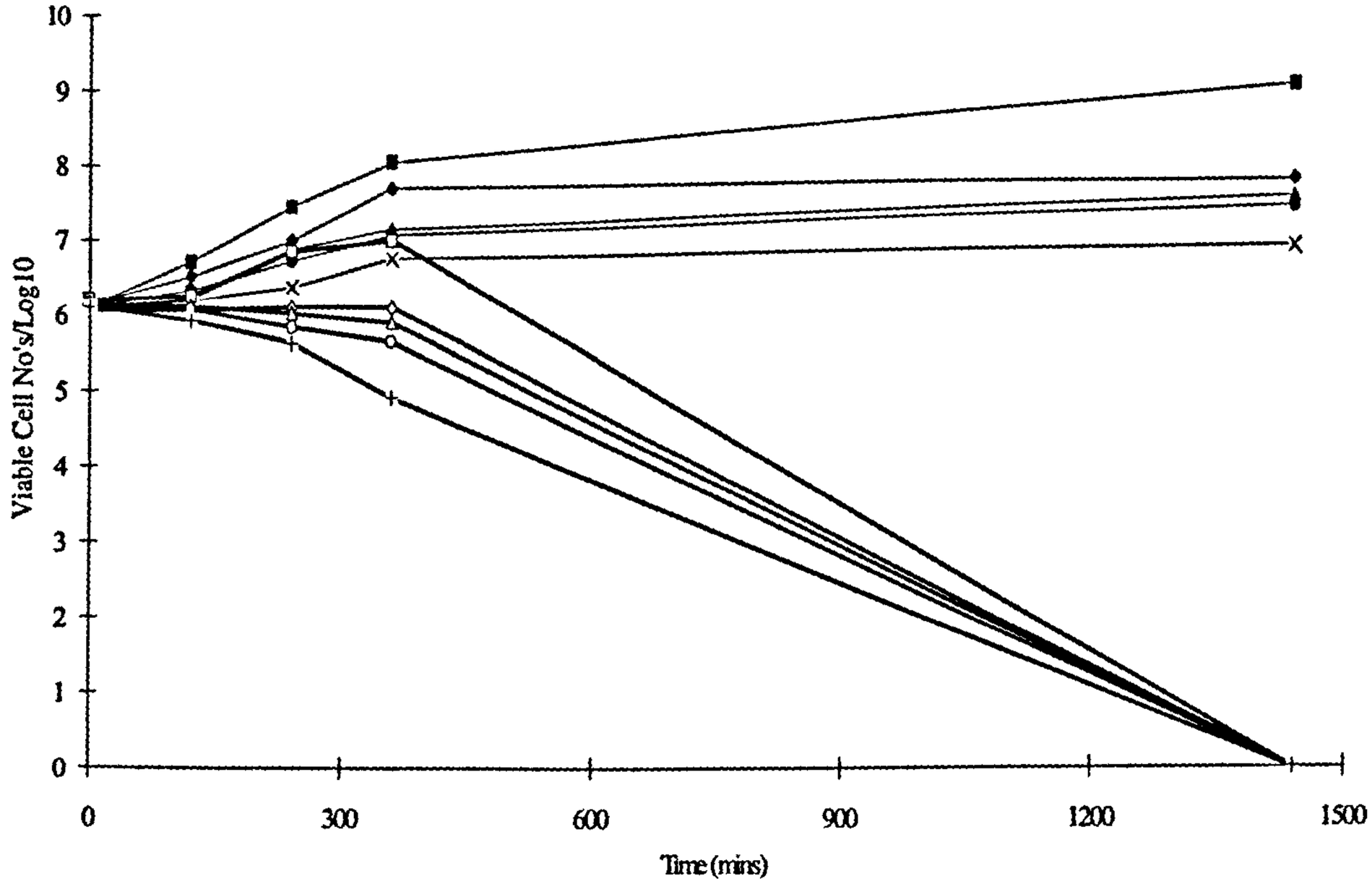
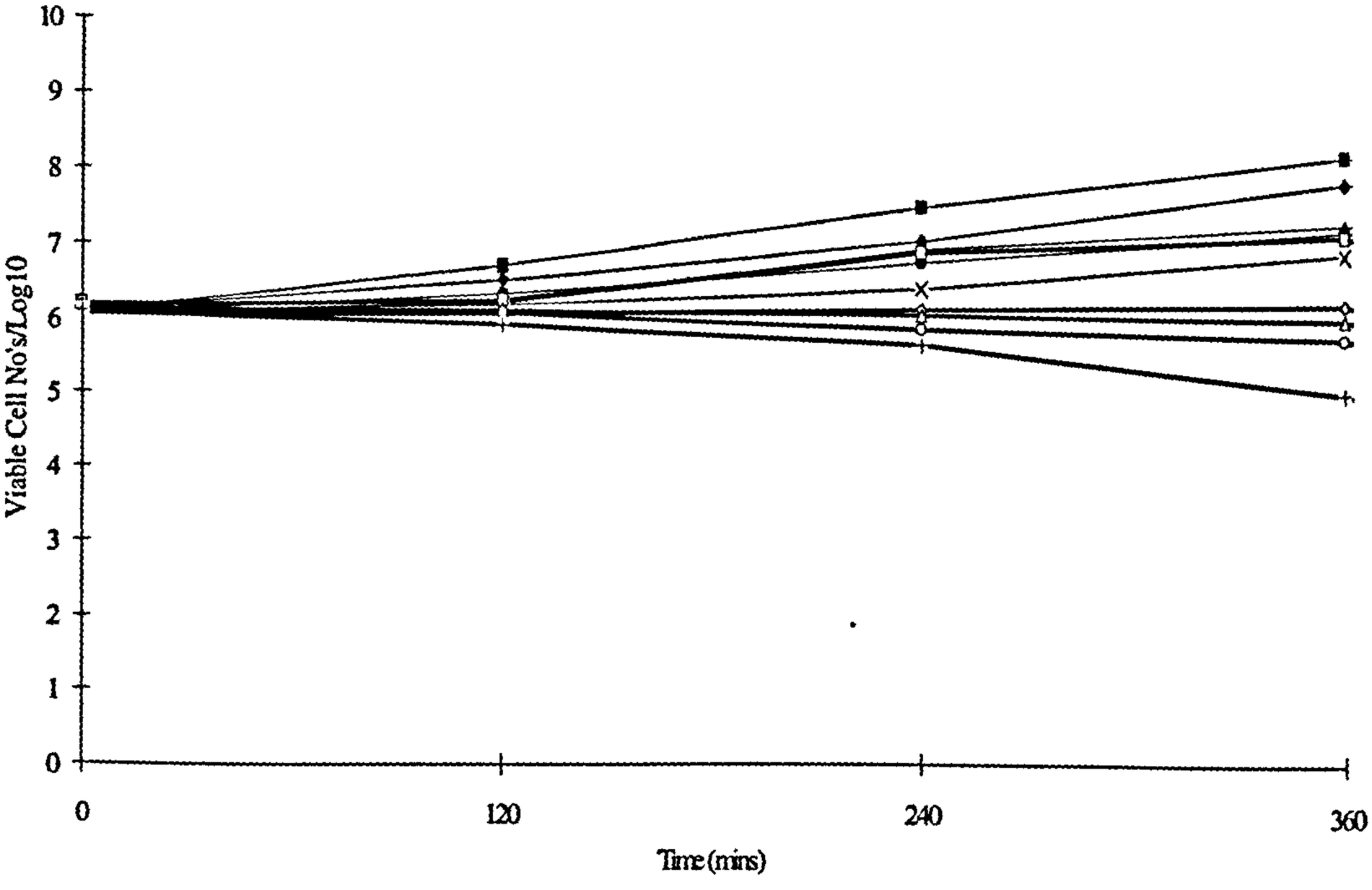


Figure 3.5.2d



During the first 360 minutes (Figure 3.5.2d), it was observed that in the absence of G.O both organisms proliferated at similar initial growth rates to those seen in pure culture. The *L. acidophilus* cells continued to proliferate, with the final population size being greater than when grown in pure culture. In contrast following a steady rise in *L. monocytogenes* viable cell populations for the first 360 minutes after inoculation dramatic decline to complete kill at 24 hours occurred.

In the presence of G.O, differences in the effect on the organism in the mixed culture to those of pure culture studies were observed; 1) As with the pure culture studies in the presence of G.O, 'lag' phases were observed for *L. acidophilus*, however these were not as marked as in pure culture; 2) At all G.O concentrations (including 0.34mg/ml), the cells proliferated slightly faster than in the pure culture study; 3) The initial death rates of *L. monocytogenes* were similar to those in pure culture; 4) For *L. monocytogenes*, over the first 360 minutes the lowest G.O concentration of 0.04mg/ml resulted in an inhibition of growth while at concentrations greater than 0.08mg/ml, cell death commenced immediately after inoculation; 5) By 24 hours all G.O concentrations were sufficient to produce complete kill of the *L. monocytogenes* population.

Thus in the presence of *L. acidophilus* the net antimicrobial action against *L. monocytogenes* seen in the presence of G.O is substantially increased. It is known that *L. acidophilus* produce bacteriocins effective against *L. monocytogenes* (Vandenbergh, 1993) in addition to compounds such as H_2O_2 (Appella *et al.*, 1992). These may account for the loss of *L. monocytogenes* cell viability in the mixed culture not observed in the pure culture studies. Alternatively the production of organic acids by *L. acidophilus* and therefore a reduction in pH may also be partly or wholely attributable.

3.5.3 The Ability Of G.O To Exert Antimicrobial Activity Within A "Real" Gut Fluid Model (Ileostomy Fluid, IF)

The model systems previously described are those of either (favourable) growth media or synthetic gut fluids. Whilst these probably provide a fair approximation of the *in vivo* environment, real-gut fluid is yet more complex, especially in terms of solids. Therefore the next step was to assess the effect of G.O in a fluid derived from the intestine, ileostomy fluid (IF).

The particular advantage of using IF was to establish whether the presence of G.O in the intestinal tract can exert its effect against incoming food-borne pathogens in the presence of, and whilst allowing, the natural intestinal bacteria to continue to thrive.

Initial observations of the ileostomy effluents provided some insight into the properties of the IF samples. It was thought that the effluents would be aqueous, that is, having not passed through the colon and not undergone dehydration, however in most cases the effluents were viscous, containing approximately 30-50% solid material. It was also noted that the effluents varied in colour ranging from green to dark brown, this was thought to be due to their bile content. Sample volumes also varied from 20-150ml depending upon the timing of previous emptying of the ileostomy bag.

The 1-10 year post-operation samples collected exhibited mean pH values of 6.21±1.0, which was comparable to that of published data (Finegold *et al*, 1970). The amount of available oxygen present in the pooled samples used for viability studies was also determined using an oxygen electrode pre-calibrated with nitrogenated water. A reading of 0.00 was obtained in all cases, indicating that the dissolved O_2 present in the pooled IF samples was less than 1μM, the detection limit of the O_2 electrode. This result indicates that the subsequent experiments with IF were essentially anaerobic and that any O_2 diffusing into the system from the IF:AIR interface was likely to be rapidly removed by facultative anaerobes present in the sample.

Initial microbial analyses were performed on ileostomy effluents obtained from 1-2 day post-operation patients. This resulted in a microbial count of $2x10^6$ organisms/g effluent, whilst those from 6-7 day post-operation patients were in the region of $4x10^6$ organisms/g effluent, both of which show a lower viable count compared to the samples obtained from the more established 1-10 years post-operation patients which yielded $6x10^8$ organisms/g effluent. This difference was probably due to A) the disturbance of the microbial flora after operation OR B) influence of drug treatment (See Discussion p233).

It was therefore decided to monitor the microbial composition of ileostomy effluents obtained from 5 post-operation (1-10 years) patients (See Table 3.5.3a), taken at the same time on the same day each week over a period of 4 weeks. Determination of the aerobic and anaerobic bacterial count of the ileostomy effluents was performed in triplicate on tryptone soy agar (TSA) and blood agar (BA) plates incubated under both aerobic and anaerobic incubation conditions at 37°C.

The results indicated that microbial counts varied not only between different patients (mean ranges from $1.78x10^8$-$1.85x10^9$) but from week to week with the same patient (for example patient No. 1 counts range from $1.74x10^7$-$1.84x10^8$). As the samples were collected the same time of day each week and microbial analysis was performed in all cases 1 hour after collection, the differences may have resulted from the length of time the effluent remained within the ileostomy bag. This will depend upon the patient's emptying of the bag, which given a long enough period of time may allow for an overgrowth of one particular organism of other types of organism according to the prevailing conditions for growth. Diet could also produce substantial changes as the nature of the digesta are liable to affect the microbial environment.

Despite this great potential variation, the mean results of approximately $6x10^8$ organisms/g ileostomy effluent obtained here from the TSA plates were comparable to those of published data (Gorbach *et al.*, 1967c; Scarpino *et al.*, 1969 and Finegold *et al.*, 1970),

although it can be seen that different microbial counts were obtained with the different isolation media.

Thus it was noted that in some cases slightly higher microbial counts were obtained on BA plates ($1x10^9$ organisms/g effluent). It was thought that the difference in counts could be due to the presence of strict anaerobes, such as *Bacteroides spp* (whose distinct smell was detected), however no such bacteria were isolated on the BA plates, supporting the findings of Finegold *et al.* (1970), which indicated that virtually all organisms encountered in the GI tract are facultative organisms, growing well under both aerobic and anaerobic conditions.

Biochemical data obtained from API identification strips indicated that *E. coli* was the most established organism within the ileostomy effluents, identified as the main organism isolated on both BA and TSA plates. The other organisms identified included; *Staphylococcus aureus, Staphylococcus epidermidis, Staphylocoocus haemolyticus, Xanthomonas maltophilia, Bacillus spp* (five organisms not commonly found in the GI tract, which probably entered the effluent as it passed out through the stoma), *Vibrio vulnificus, Vibrio alginolyticus* (*parahaemolyticus*), *Klebsiella pneumoniae, Klebsiella oxytoca* (four organisms commonly found in the GI tract) and *Proteus mirabilis* (commonly isolated from clinical specimens).

Table 3.5.3a Initial Microbial Analysis

Total No. of viable cells/g of sample

	Ileostomy Samples				
	Patient No.				
Week 1 Incubation Conditions Aerobic 37°C	1	2	3	4	5
TSA	$1.30x10^7$	$1.90x10^7$	$1.71x10^8$	$8.23x10^7$	-
MC	$1.56x10^7$	$1.35x10^8$	$7.20x10^7$	$4.10x10^7$	-
Anaerobic 37°C					
BA	$2.36x10^7$	$6.63x10^8$	$1.73x10^8$	$3.96x10^7$	-
Week 2 Incubation Conditions Aerobic 37°C	1	2	3	4	5
TSA	$3.60x10^7$	$2.76x10^8$	-	$5.33x10^7$	$1.80x10^9$
MC	$5.40x10^7$	$1.77x10^8$	-	$1.15x10^8$	$1.85x10^9$
Anaerobic 37°C					
BA	$9.16x10^8$	$1.15x10^9$	-	$6.56x10^7$	$4.53x10^9$
Week 3 Incubation Conditions Aerobic 37°C	1	2	3	4	5
TSA	$1.01x10^8$	$7.50x10^9$	-	$5.26x10^8$	$6.60x10^8$
MC	$1.40x10^8$	$3.54x10^9$	-	$2.66x10^8$	$5.10x10^8$
Anaerobic 37°C					
BA	$3.13x10^8$	$8.30x10^9$	-	$5.13x10^7$	$7.90x10^8$
Week 4 Incubation Conditions Aerobic 37oC	1	2	3	4	5
TSA	-	$1.26x10^8$	$7.13x10^7$	$7.31x10^8$	$1.77x10^8$
MC	-	$1.44x10^7$	$9.96x10^7$	$3.06x10^8$	$1.13x10^8$
Anaerobic 37oC					
BA	-	$4.07x10^8$	$9.66x10^8$	$7.33x10^8$	$9.45x10^8$
Mean Values (S.D) Incubation Conditions Aerobic 37°C	1	2	3	4	5
TSA	$5.00x10^7$ ($\pm 4.4x10^6$)	$1.98x10^9$ ($\pm 2.2x10^9$)	$1.21x10^8$ ($\pm 3.5x10^7$)	$3.48x10^8$ ($\pm 5.3x10^7$)	$8.79x10^8$ ($\pm 4.4x10^7$)
MC	$6.98x10^7$ ($\pm 6.0x10^6$)	$9.66x10^8$ ($\pm 9.7x10^8$)	$8.58x10^7$ ($\pm 3.4x10^7$)	$1.82x10^8$ ($\pm 1.4x10^8$)	$8.24x10^8$ ($\pm 8.8x10^7$)
Anaerobic 37°C					
BA	$4.17x10^8$	$2.63x10^9$	$5.69x10^8$	$2.22x10^8$	$2.08x10^8$
	($\pm 3.8x10^7$)	($\pm 1.4x10^9$)	($\pm 5.4x10^7$)	($\pm 1.5x10^8$)	($\pm 4.0x10^7$)

The physical consistency and inherent turbidity of IF was such that MIC determinations could not be performed. Instead initial viability studies were performed in which the population of selected normal microflora present in the IF plus *L. monocytogenes* inoculated into it, was monitored with respect to time in the presence of various G.O concentrations. The aim of this experiment was two-fold to assess the effect of G.O on; 1) the natural bacteria present in the IF sample, AND 2) the *L. monocytogenes* cell population inoculated into it. These viability studies were repeated on three separate occasions and are presented as the means of the three experiments (Figures 3.5.3b-e). These studies utilised the following media; TSA, MC (Mac Conkeys Agar), BA and LSA, so as to provide valid enumeration of the bacteria present in the ileostomy samples. Thus TSA (aerobic) and BA (anaerobic) measure the total number of bacteria present in the sample capable of growing on either media including *L. monocytogenes*, whilst MC (aerobic) measures most enteric bacteria but not *L. monocytogenes* and LSA would measure total *L. monocytogenes* levels.

Figure 3.5.3b: The Effect Of G.O On IF Inoculated With *L. monocytogenes* (433) On TSA Plates

Figure 3.5.3c: The Effect Of G.O On IF Inoculated With *L. monocytogenes* (433) On BA Plates

Serial two-fold dilutions of G.O in IF were prepared in tin foil capped boiling tubes to a total volume of 10ml. To each tube 20µl of an overnight culture of *L. monocytogenes* (433) was added as an inoculum. The tubes were incubated at 37°C in a water bath. Viable counts were performed with respect to time using the spread plate technique.

Note: The counts of bacteria on the TSA plates indicate the **total numbers of bacteria** present in the tubes, that are able to grow on TSA under aerobic incubation conditions.

Note: The counts of bacteria on the BA plates indicate the **total numbers of bacteria** present in the tubes, that are able to grow on BA under anaerobic incubation conditions.

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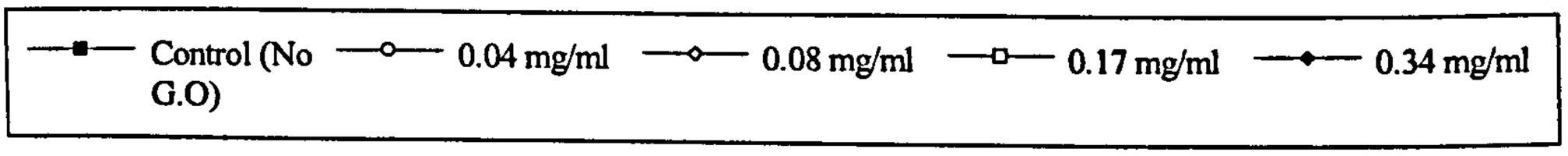

Figure 3.5.3 b

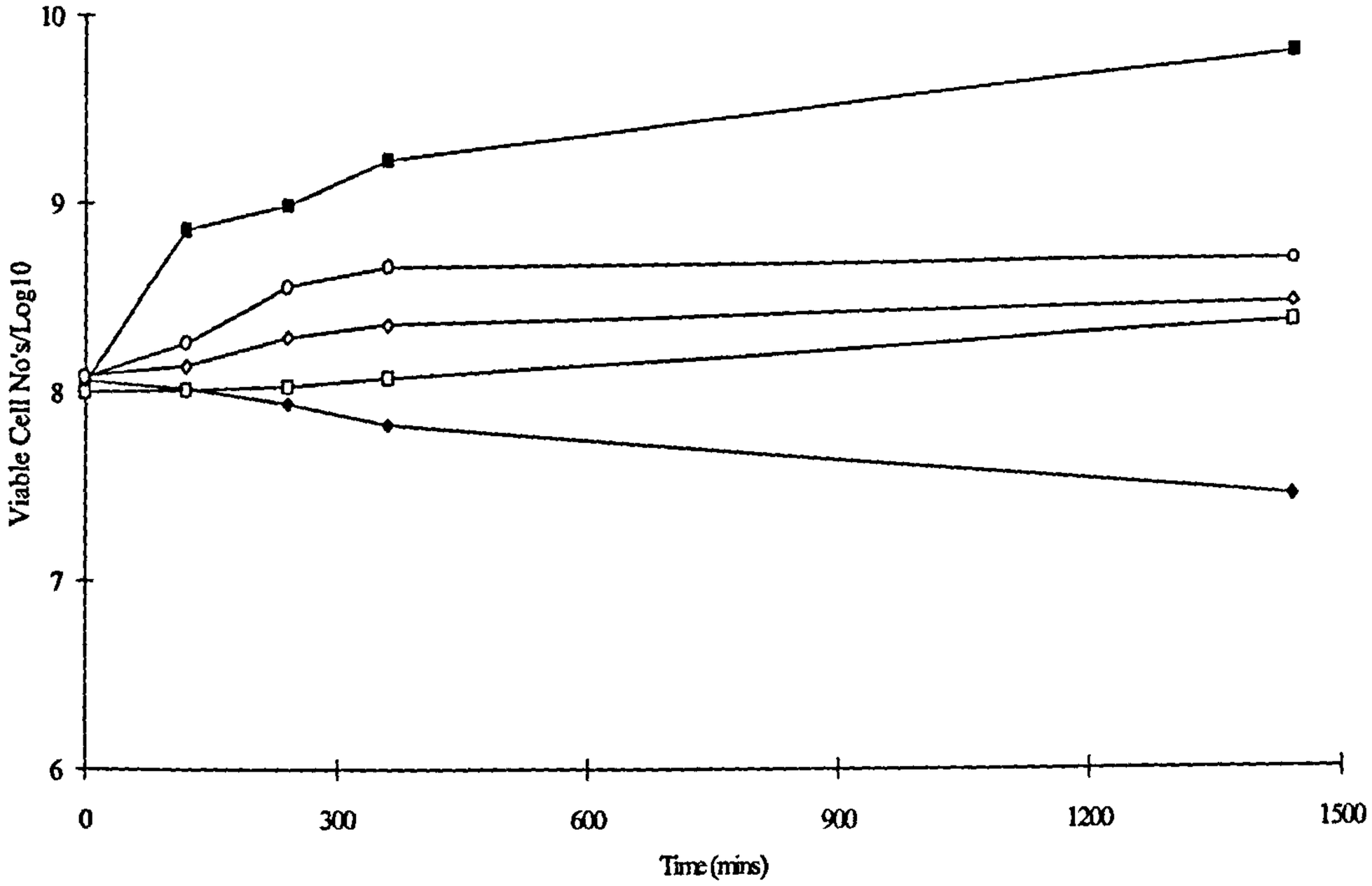

Figure 3.5.3 c

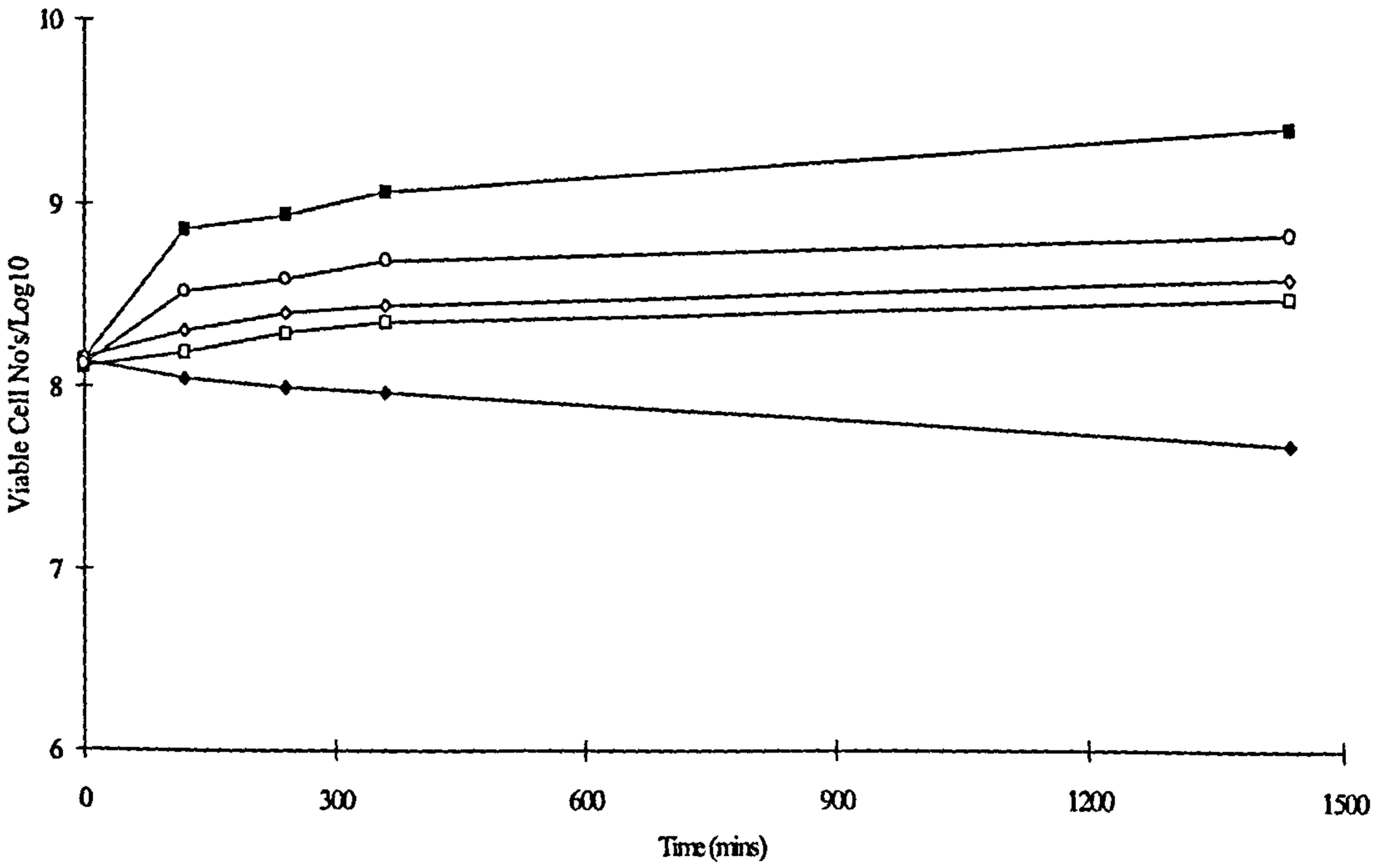

Figure 3.5.3d: The Effect Of G.O On IF Inoculated With *L. monocytogenes* (433) On MC Plates

Figure 3.5.3e: The Effect Of G.O On IF Inoculated With *L. monocytogenes* (433) On LSA Plates

Serial two-fold dilutions of G.O in IF were prepared in tin foil capped boiling tubes to a total volume of 10ml. To each tube 20µl of an overnight culture of *L. monocytogenes* (433) was added as an inoculum. The tubes were incubated at 37°C in a water bath. Viable counts were performed with respect to time using the spread plate technique.

Note: The counts of bacteria on the MC plates indicates the **total numbers of bacteria** present in the tubes, that are able to grow on MC under aerobic incubation conditions.

Note: The counts of bacteria on the LSA plates indicates the **total numbers of** *L. monocytogenes* present in the tubes.

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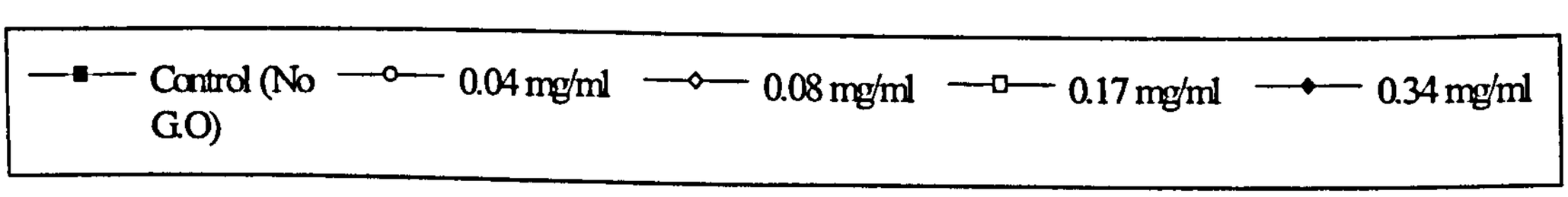


Figure 3.5.3 d

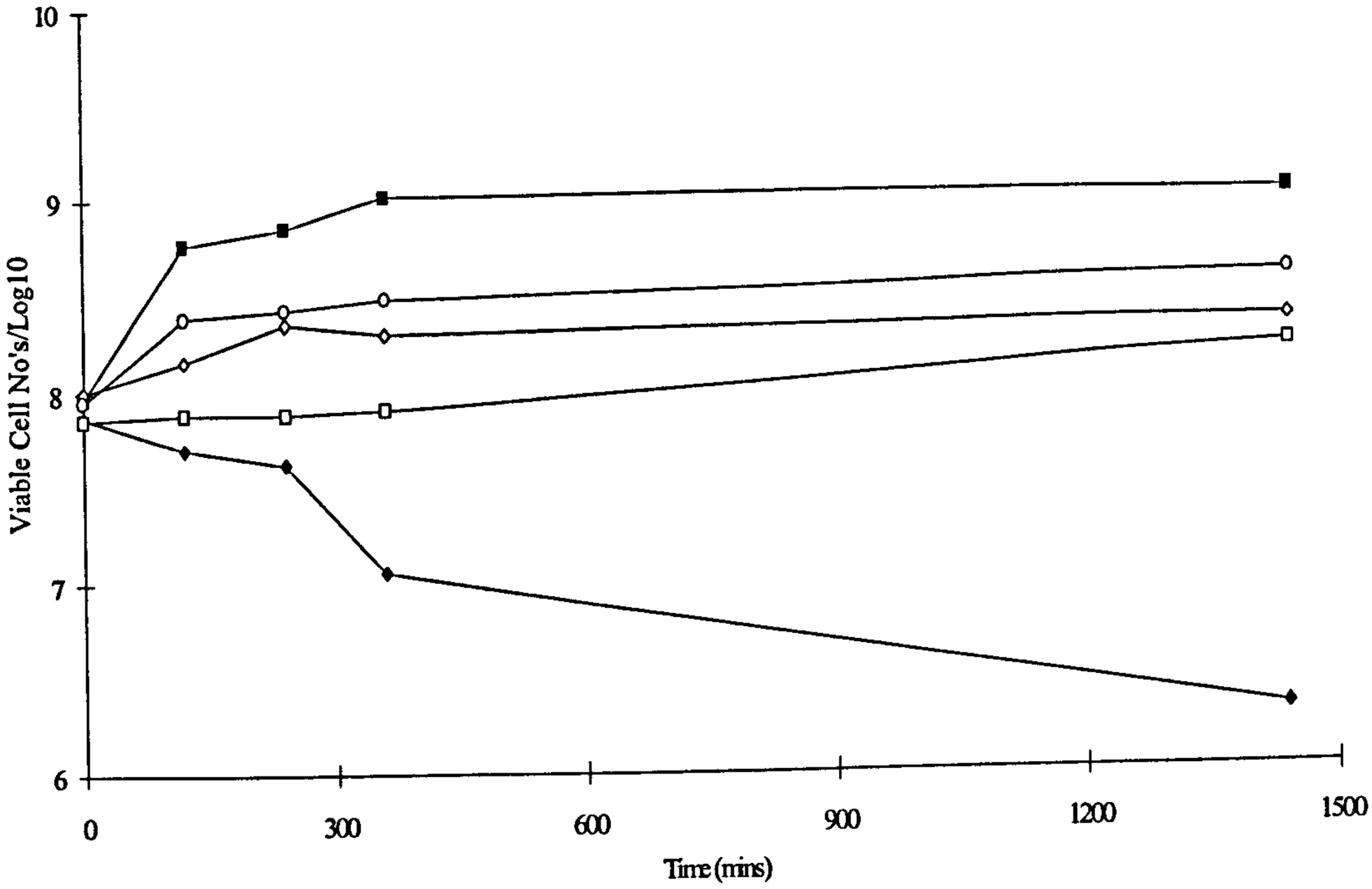
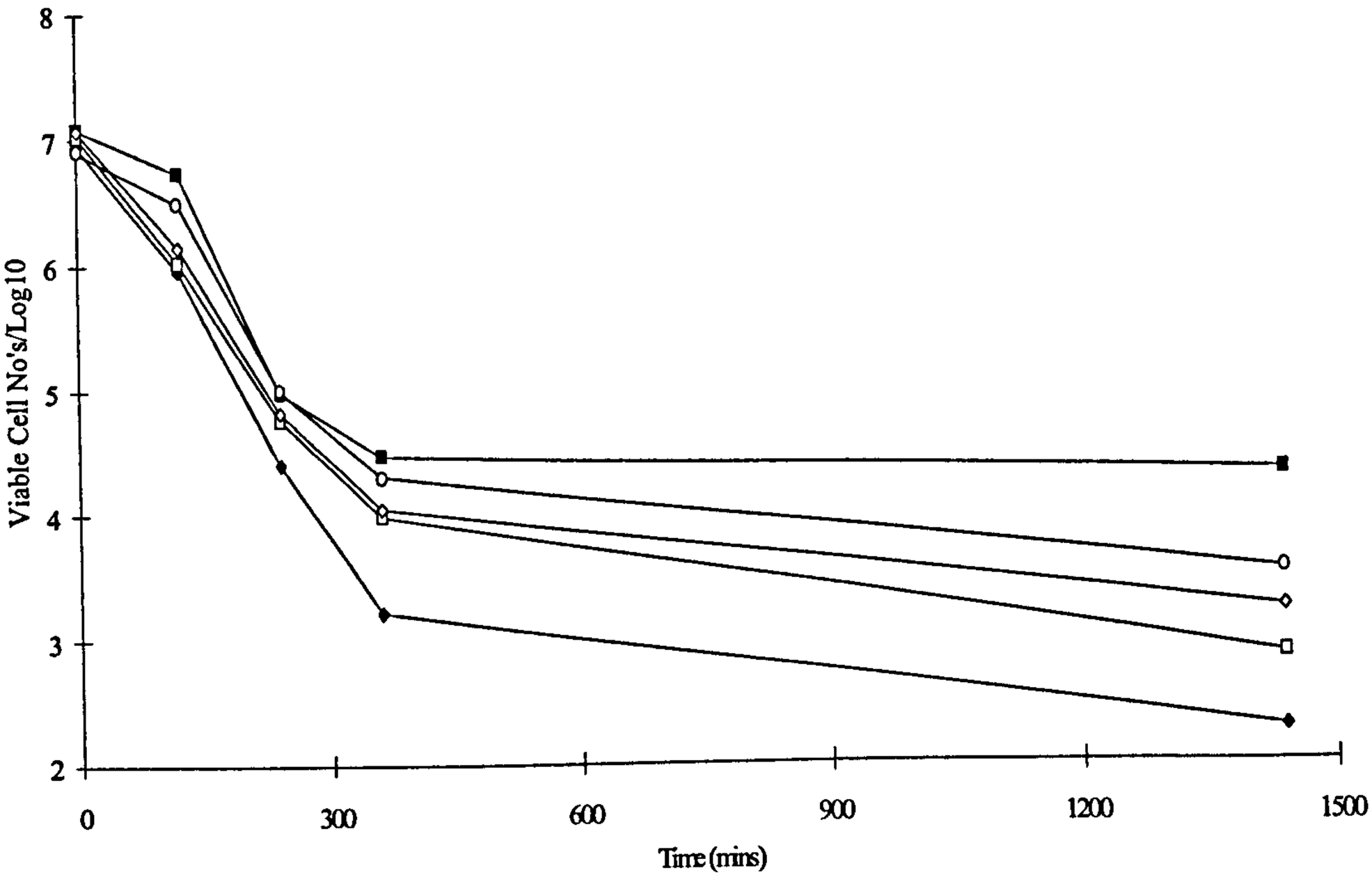


Figure 3.5.3 e



From the graphs it can be seen that the overall pattern of G.O antimicrobial effectiveness present in the IF is similar to that previously obtained in TSB, MRS, SIF and CIF. Thus G.O is shown to be as effective antimicrobially against *L. monocytogenes* in the "real" gut fluid as it is in microbiological media and gut-fluid modelling media.

On TSA plates (Figure 3.5.3b), a similar response of the total mixed bacterial population was observed to that of the pure culture studies (Figure 3.2a), that is, in the control (No G.O) the bacteria steadily increase in numbers while in the presence of various G.O concentrations, it can be seen that the response of the viable cell population is proportional to the concentration of G.O present.

Comparison of TSA and BA plates (Figure 3.5.3c) indicated similar results. It was hoped that the BA plates would isolate any strict anaerobic bacteria present in the IF, however it appears from the rather similar plate counts that only facultative bacteria are present. Comparison of the viable counts obtained on the two media indicated that anaerobic incubation conditions for the BA plates slightly inhibited the growth of these bacteria, or that certain organisms do not grow/survive well on BA plates.

Comparison of the TSA to MC (Figure 3.5.3d) plates in terms of the total viable counts indicated some differences between the two media types; 1) inhibition of growth of the isolated bacteria was more evident on the MC plates AND 2) at 0.34mg/ml G.O the number of bacteria growing on MC plates declined more rapidly and to a greater extent than those growing on TSA or BA (anaerobic) plates, indicating that this medium does not support the growth of the surviving bacteria as well as either TSA or BA.

These differences are probably due to the fact that TSA is a broad based medium used for identification of a wider range of microorganisms whereas MC is a selective medium used mainly for the isolation and identification of enteric organisms. In particular, MC contains a carbon source of peptone which may be inhibitory to some

intestinal microflora, in addition, MC also contains known potentially inhibitory components such as sodium chloride and bile salts. The MC plate results imply that 0.34mg/ml G.O selectively alters the natural microbial composition of the ileostomy effluent.

By comparing the TSA and LSA plates it can be seen that with the starting inoculum of *L. monocytogenes* used, at G.O concentrations lower than 0.34mg/ml, a decrease in the *Listeria* population (Figure 3.5.3e) was observed whilst the resident bacteria of the IF (Figure 3.5.3b) remained at a stable population level. *L. monocytogenes* could be easily identified on LSA plates since it is the only organism to produce a brown pigment surrounding the colony. Other organisms grow on LSA plates but do not produce the characteristic pigment.

On the LSA plates (Figure 3.5.3e) it was observed that a decrease in the viable cell population of *L. monocytogenes* occurred with time in the control (no G.O). Greater reductions showing a similar pattern of decline are obtained in the presence of various G.O concentrations. Possible explanations for an intrinsic IF component in the decreases observed are; 1) the composition of the IF in terms of biochemical physiology, such as, concentration of inhibitory components such as bile AND/OR 2) the presence of one or more of the other bacteria in the IF having an antagonistic effect on the *L. monocytogenes.*

Although the actual composition of the IF has not been analysed as part of this study, published data on the composition of ileostomy samples indicates that bile is present (at an average concentration in the jejunum/ileum of 10mmol/L) (Finegold *et al.* 1970) and as stated previously *L. monocytogenes* is sensitive to the presence of bile (p119), thus providing some support for this explanation.

The rapid increase in the resident bacterial population of the IF from 0-120 minutes (Figure 3.5.3b), compared to the decrease in *L. monocytogenes* population after the same

period (Figure 3.5.3e), provides some evidence to support the second explanation. Complementary evidence is provided by results obtained in Section 3.5.2, which shows that organisms such as *L. acidophilus* (an organism which occurs naturally in the intestinal tract) produce biocins that are effective against *L. monocytogenes.*

To examine these hypotheses, a number of experiments were performed in which the viability of *L. monocytogenes* in the absence of G.O was monitored in various preparations of IF (See Figures 3.5.3f, g). These experiments were also designed in order to evaluate the effect of G.O in IF, that is, with other hypothesised antimicrobial effects (such as bile and Lactobacillary biocins) removed since they partly mask the overall effect of G.O.

Figure 3.5.3f: The Effect Of IF Preparations On The Viability of *L. monocytogenes* On BA Plates

Figure 3.5.3g: The Effect Of IF Preparations On The Viability Of *L. monocytogenes* On LSA Plates

Four preparations of the IF were set up for analysis:-

1) whole ileostomy fluid (not autoclaved)

2) whole ileostomy fluid (autoclaved)

3) supernatant (not autoclaved)

4) supernatant (autoclaved)

The supernatant was obtained by centrifugation of the whole ileostomy fluid at 14,000 rpm at 4°C for 30 minutes. 20ml of each preparation was placed into tin foil capped sterile boiling tubes and 40µl of an overnight culture of *L. monocytogenes* added. Viable counts were performed at time intervals using the spread plate technique onto BA plates incubated under anaerobic conditions and LSA plates incubated under aerobic conditions.

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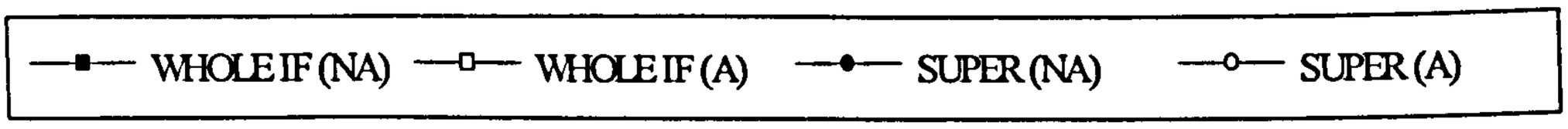


Figure 3.5.3 f

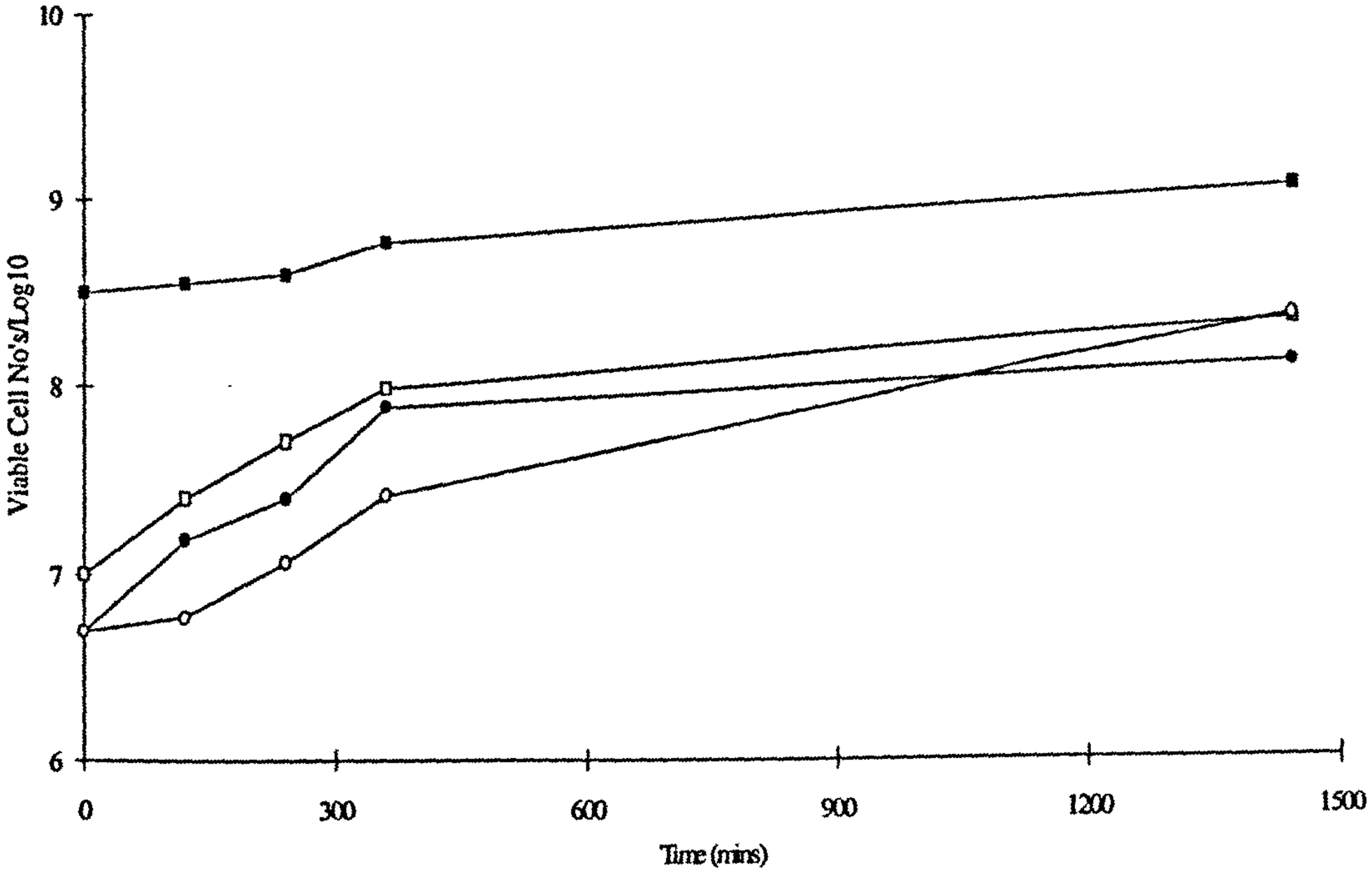
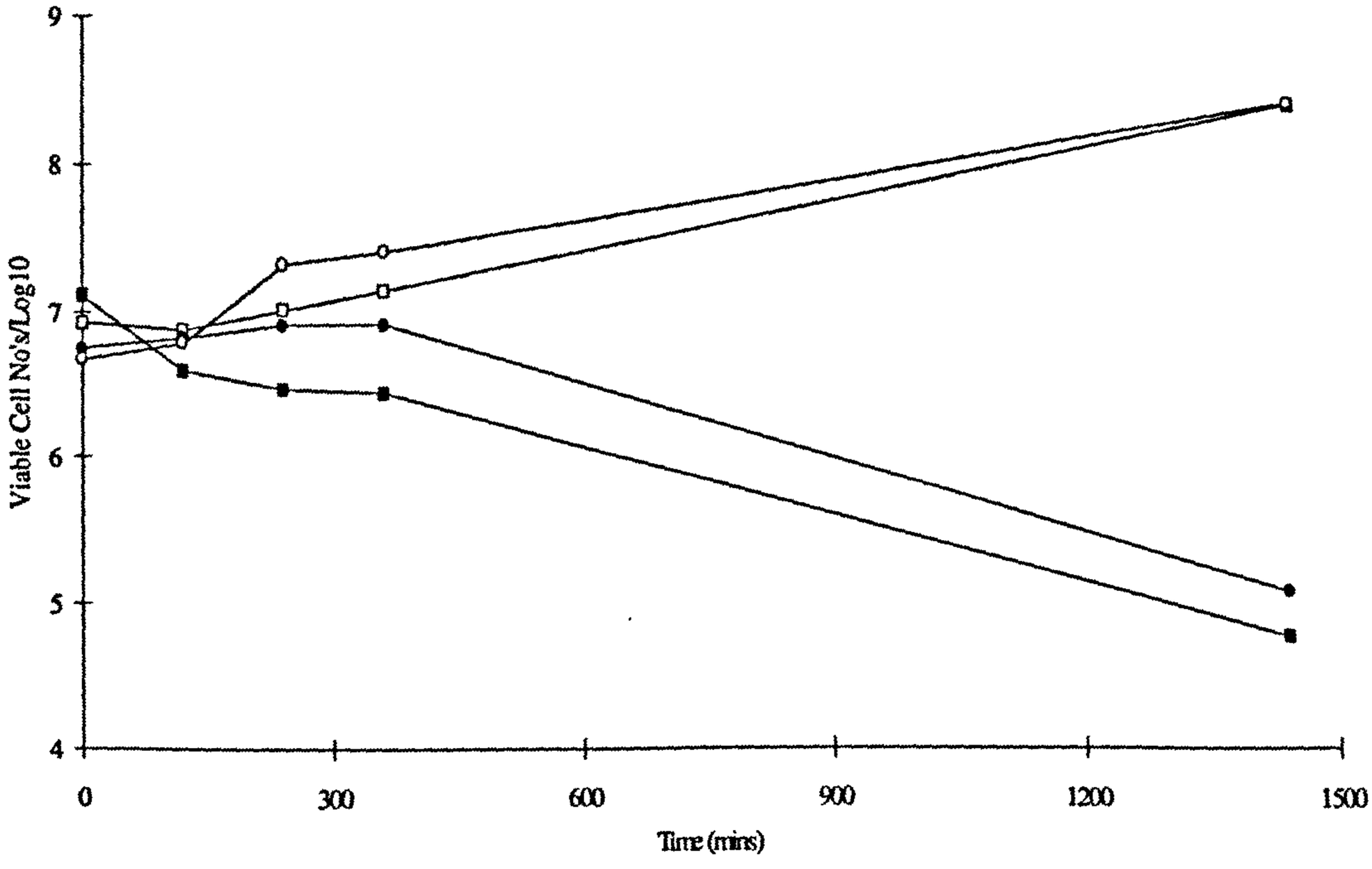


Figure 3.5.3 g



From the graphs it can be seen that the preparation/state of IF has an influence on the growth and viability of *L. monocytogenes* cells. On the BA plates (Figure 3.5.3f) the viable counts obtained from both the non-autoclaved fluid preparations represent both the original organisms present in IF plus added *L. monocytogenes.* On the LSA plates (Figure 3.5.3g) the counts observed represent the *L. monocytogenes* cells only. In the autoclaved preparations the numbers of bacteria isolated on both BA and LSA are assumed to represent the added *L. monocytogenes* cells only.

By comparing the different preparations it can be seen that the graphs complement each other quite closely. In whole IF not autoclaved, it can be seen that the natural bacteria of the IF fluid are present at a level (closed squares, Figure 3.5.3f) which have a significant inhibitory effect on the viability of *L. monocytogenes* but which decreases with respect to time (closed squares, Figure 3.5.3g). In the autoclaved whole fluid and supernatant preparations, in the absence of the natural bacteria, *L. monocytogenes* proliferated readily (open symbols, Figures 3.5.3f and g). However the *L. monocytogenes* cells do not reach the same high population density after 24 hours in autoclaved whole IF (open squares, Figure 3.5.3g) as is seen for the whole non-autoclaved IF sample (closed squares, Figure 3.5.3f). This could mean that another factor present in the IF has an influence on the growth and viability of the *Listeria* cells, such as the biochemical physiology of IF.

The purpose of centrifugation was to try to remove the natural bacteria present in IF sample without causing any chemical and thence physiological changes resulting from the high temperatures employed in autoclaving. Figure 3.5.3f (closed circles compared to closed squares), shows that centrifugation does not completely remove all the resident bacteria but does result in a hundred fold reduction in cell population numbers, which increase over the 24 hour period. This may explain the initial increase in *L monocytogenes* cell population size seen prior to its rapid decrease. If so the secondary decrease in *L. monocytogenes* referred to (closed circles, Figure 3.5.3g) could result from an antagonistic effect of the IF bacteria.

The relationship between G.O concentration and *L. monocytogenes* death rate within IF was compared to that in other media (See Figure 3.5.3h). Here the initial cell death rate of *L. monocytogenes* in IF is well defined, compared with that in CIF and TSB (Section 3.5.1). This is probably due to the addition of the anti-*Listeria* factors referred to in the explanations 1 and/or 2 (p161). It may well be that one of the problems of using whole IF samples is that the initial effect of G.O is hard to evaluate due to a masking effect of other inhibitory effects of IF.

The initial viability experiments described above were repeated on ten separate occasions, each time using pooled IF samples from different patients (not shown). Although some differences were observed in terms of the total viable counts of IF microflora determined from the initial microbial analysis (Table 3.5.3a), no difference in the pattern of antimicrobial effectiveness of G.O was observed. It should be stated however, that the reproducibility of such experiments is difficult to maintain, as each patient has a variety of different bacteria present, in terms of numbers and types, which varies according to the length of time after post-operation.

Figure 3.5.3h: Comparison Of Initial Cell Death/Growth Rates Of *L. monocytogenes* (433) In TSB, CIF & IF

The source of data for this graph was derived from the initial cell death/growth rates determined from Figures 3.5.1j, k and Figure 3.5.3e.

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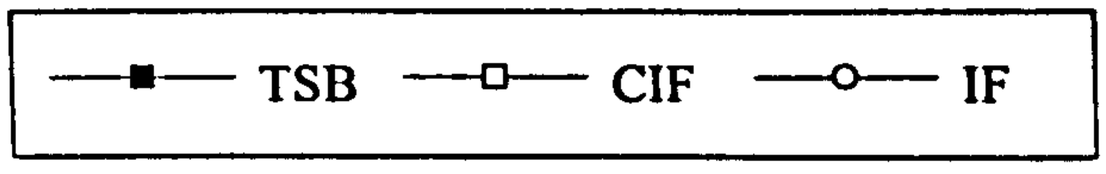
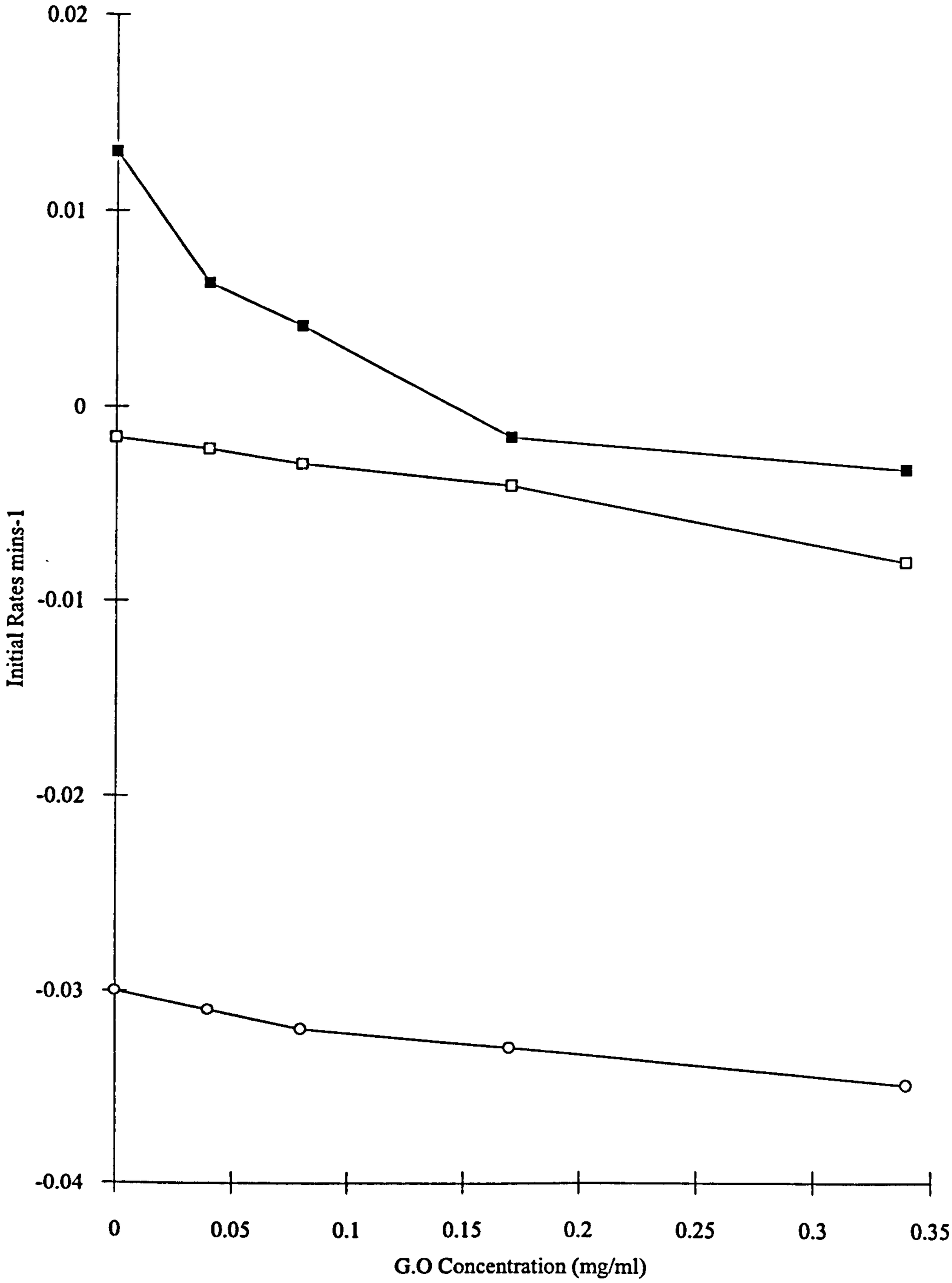


Figure 3.5.3 h


0.02
0.01
0
-0.01
-0.02
-0.03
-0.04
Initial Rates mins-1
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
G.O Concentration (mg/ml)

SECTION 2 - Analysis Of The Garlic Products

3.6 Quantitative/Qualitative Analysis

3.6.1 Quantitative Estimation Of The Allicin Content In Freeze-Dried G.P

3.6.1.1 Spectrophotometric Determination Of The Allicin Content In G.P

Quantification of the allicin content of G.P by ammonia and pyruvate assays provided similar estimates for the allicin content (derived from alliin) in 24 replicate samples of the stock preparation (1g G.P in 10ml H_2O), called "10% G.P". Analyses of the allicin content of the centrifuged supernatant (aqueous preparation used for the microbial work) was determined (in terms of ammonia and pyruvate estimates) to be approximately 85-89% of whole stock G.P in the water preparation. Analysis of the pellet formed by centrifugation after being resuspended in an equal w/v ratio of water, accounted for approximately a further 13%. This indicates that some of the allicin remains within the powder pellet after centrifugation. All these determinations were statistically analysed having a constant acceptable %CV of less than 2.5.

The allicin content (as determined by the pyruvate and ammonia assay systems) of the filter sterilised aqueous 10% preparation was calculated to be approximately 11.00mg /g and 10.93mg/g G.P respectively from 12 replicate samples with a constant %CV of less than 5.

3.6.1.2 Estimation Of Vinyldithiins By GC - As A Means Of Quantifying The Allicin Content In G.P

It is well established that allicin, under the high temperature conditions of GC analysis (150-200°C) is unstable (Brodnitz *et al.*, 1971; Saito *et al.*, 1989) decomposing to two cyclic compounds known as vinyldithiins, namely 3-vinyl-4H-dithiin-1,2 and 2-vinyl-4H-dithiin-1,3, as shown in Figure 3.6.1.2c, Table 3.6.1.2a, b. The estimation of these two vinyldithiins using GC can be related to the original content of allicin (Block, 1985).

Figure 3.6.1.2a (p172) indicates the presence of 2 compounds on the chromatograph, indicating that the G.P preparation contains allicin. The ratio of 2-vinyl-4H-1,3 dithiin to 3-vinyl-4H-1,2 dithiin is approximately 3:1 (Table 3.6.1.2b). This ratio was shown to be consistent when analyses were repeated on 10 separate occasions.

Table 3.6.1.2b: Vinyldithiin Content Of G.P From GC Analysis (Figure 3.6.1.2a)

Retention Times	Vinyldithiins	[Analyte] mg/g G.P
25.10	3-vinyl-4H-1,2-dithiin	1.31
28.43	2-vinyl-4H-1,3-dithiin	4.06

Quantification of the allicin content of the G.P preparation is shown in Table 3.6.1.2c. The calculation uses dipropyl disulphide as the internal standard and the relative GC FID response factor (as explained in Appendix 4). The figures in parentheses are standard deviations of the mean. Allicin and alliin contents were calculated on the molecular balance (Yan *et al.*, 1993).

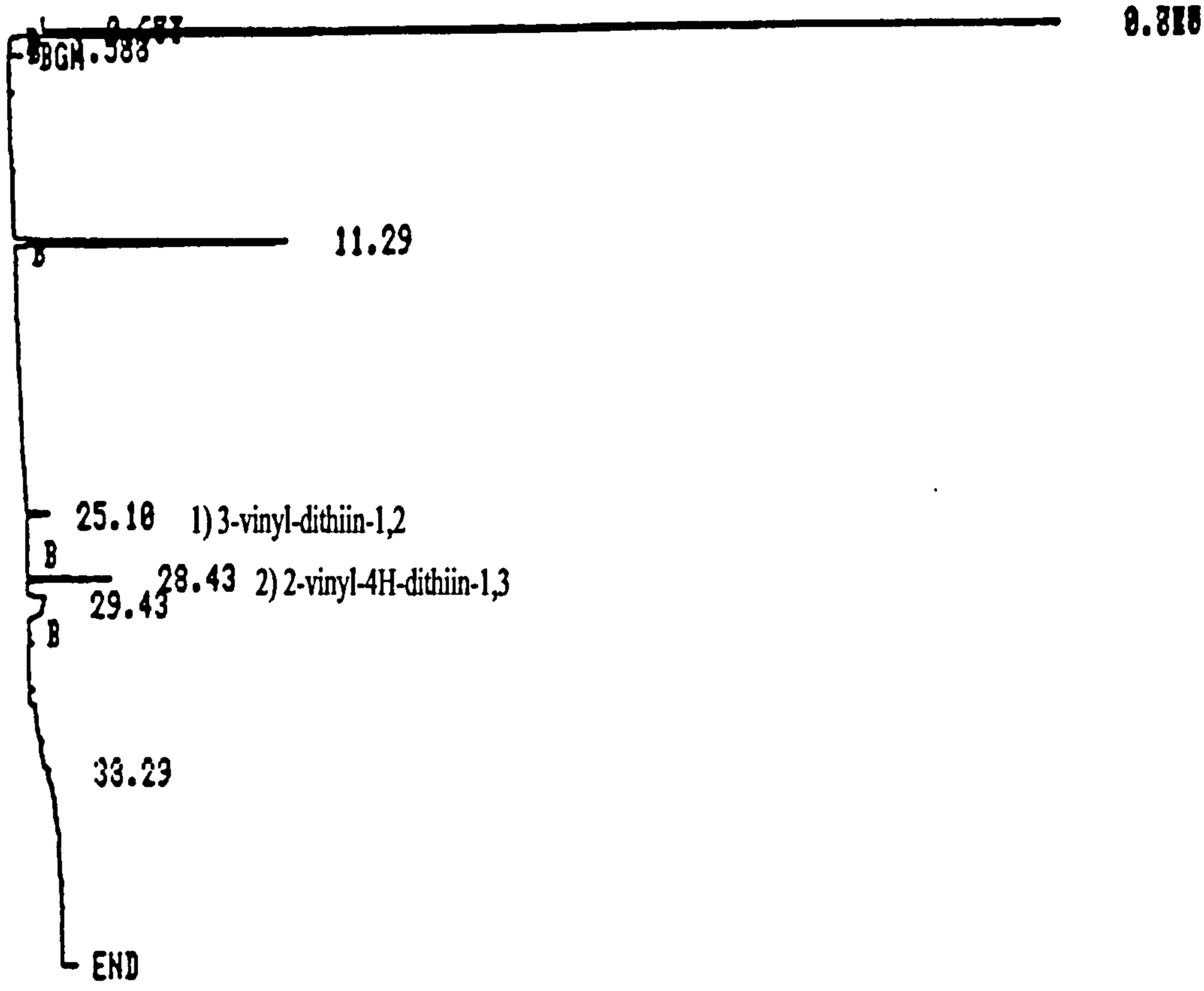
Table 3.6.1.2c: Allicin Content As Determined By GLC

Vinyldithiins (mg/g)	Allicin equivalent (mg/g)	Alliin equivalent (mg/g)
5.37 (±0.64)	6.07 (±0.54)	13.26 (±1.05)

Figure 3.6.1.2a: GC Analysis Of Vinyldithiins Derived From G.P - As A Means Of Quantifying The Allicin Content Of A G.P Preparation

1g of G.P was homogenised with 30ml water on ice for 1 minute. The homogenate was then left for 30 minutes at room temperature to allow alliin conversion to allicin. This was then centrifuged at 10,000 rpm (MSE Europa M24) for 15 minutes at 4°C. 2ml of the supernatant was then applied to a preconditioned C_{18} (SEPPAK) cartridge and the allicin eluted with 15ml of isooctane. This eluate was then heated to 100°C in a gas tight vial for 10 minutes (so that allicin converted to vinyldithiins). 2mg of the internal standard, dipropyl disulphide (retention time 11.29), was added and 0.2µl of the sample injected directly into the GC.

Figure 3.6.1.2a



3.6.1.3 Qualitative Determination Of Allicin Present In G.P By HPLC

Repeated qualitative HPLC analysis of a 10% aqueous G.P preparation to resolve allicin was performed in order to validate reproducibility. A typical HPLC trace of an aqueous G.P extract, resolving allicin (retention time 6.82) and a number of other components is shown in Figure 3.6.1.3a. Tentative identification of these components was made on the basis of the comparable results of Lawson *et al.* (1991c), these other components may be; dimethyl thiosulphinate (RT 3.21), allyl methyl thiosulphinate (RT 3.60), propyl methyl thiosulphinate (RT 4.53), allyl 1-propenyl thiosulphinate (RT 8.19) and di(1-propenyl) thiosulphinate (RT 12.14). It should be noted that quantification of allicin using HPLC could not be achieved due to a lack of suitable internal standard.

Figure 3.6.1.3a: HPLC Analysis Of Allicin From G.P

1g G.P was added to 10ml water and left to stand at room temperature for 30 minutes to allow alliin conversion to allicin. The preparation was then centrifuged at 10,000 rpm (MSE 18) for 15 minutes at 4°C. The resulting supernatant was filtered through a HPLC filter prior to injection into the HPLC system.

Conditions of HPLC:

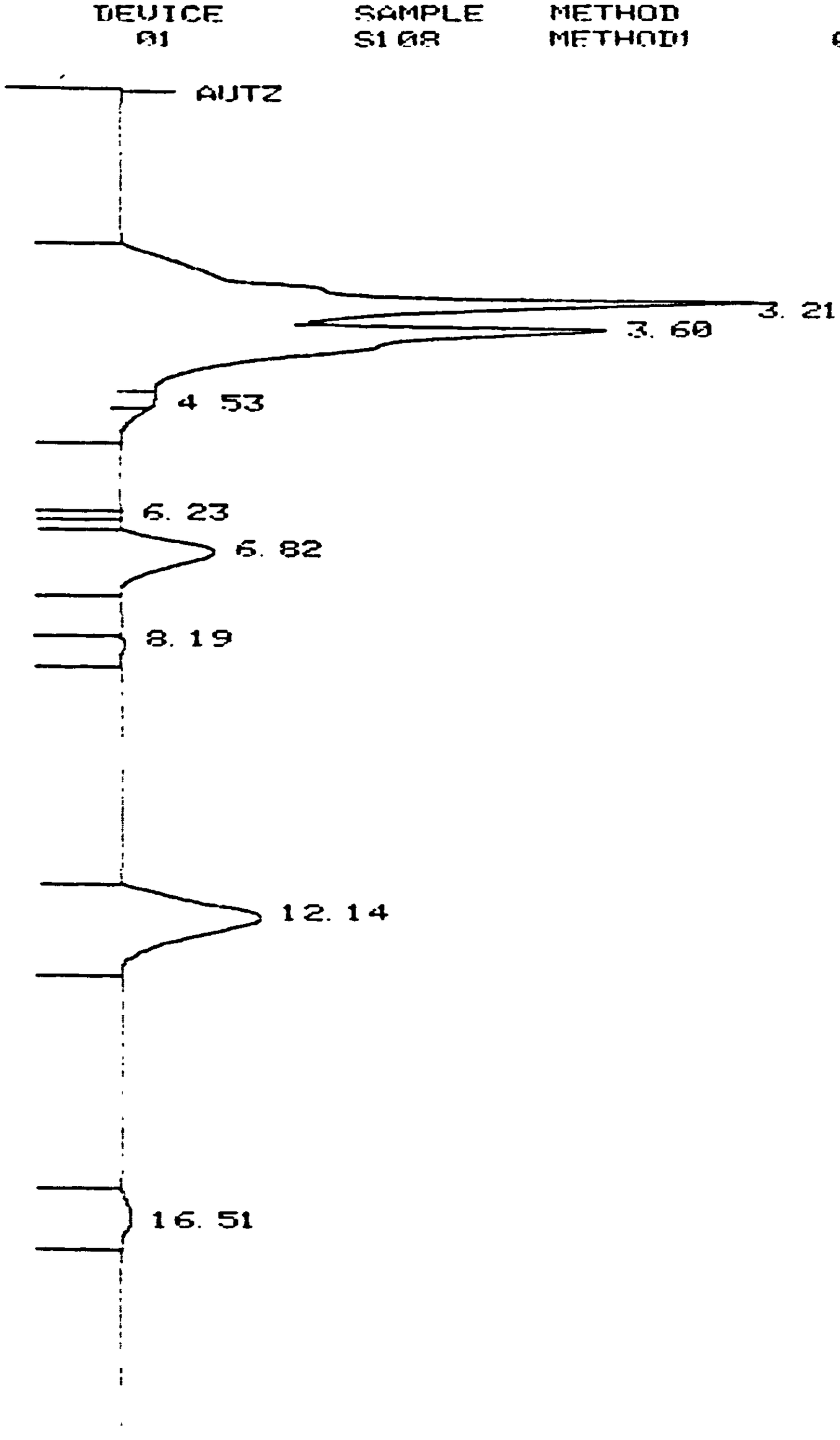
1) Mobile Phase = Methanol:H_2O (50:50)

2) Flow Rate 1.0ml/min

3) ODS2 (C_{18}) Column

4) UV detector Absorbance at a wavelength of 254nm

Figure 3.6.1.3a



3.6.2 Quantitative Estimation Of The Sulphides Present In G.O By GC

Effective separation of G.O components was achieved by gas chromatography subsequent to extraction in isooctane using the method established at Humberside University (See Methods p53). This method was repeated on ten separate occasions to determine the reproducibility of the retention times for individual G.O sulphide component peaks in order to ascertain a profile of the sulphide content that can be used as a "finger print" method of identification.

Quantification of G.O sulphides was performed using known amounts of the internal standard (IS) dipropyl disulphide (1mg/10ml), which is not reported to be present in G.O.

A typical gas chromatograph of garlic oil is shown in Figure 3.6.2a. Quantification (as determined in Appendix 5) and identification of the G.O sulphides from the trace are shown in Table 3.6.2b.

Table 3.6.2b Sulphide Content Of G.O From GC Analysis

Retention Time	Sulphides	[Analyte] mg/g G.O	% of total sulphides
2.97	(DMD) Dimethyl disulphide	9.10	1.51
3.89	(DAS) Diallyl sulphide	15.69	2.61
6.23	(MAD) Methylallyl disulphide	71.65	11.91
10.83	(DMT) Dimethyl trisulphide	24.04	3.99
11.31	(IS) Internal Standard	--	--
16.46	(DADS) Diallyl disulphide	184.29	30.65
20.46	(MATS) Methylallyl trisulphide	132.71	22.07
27.05	(DATS) Diallyl trisulphide	163.63	27.22

Figure 3.6.2a: GC Analysis Of G.O

10mg of G.O was extracted into 5ml of isooctane containing 1mg of the internal standard (dipropyl disulphide). 0.2µl of the sample was then injected directly into the GC.

Conditions Of GC:

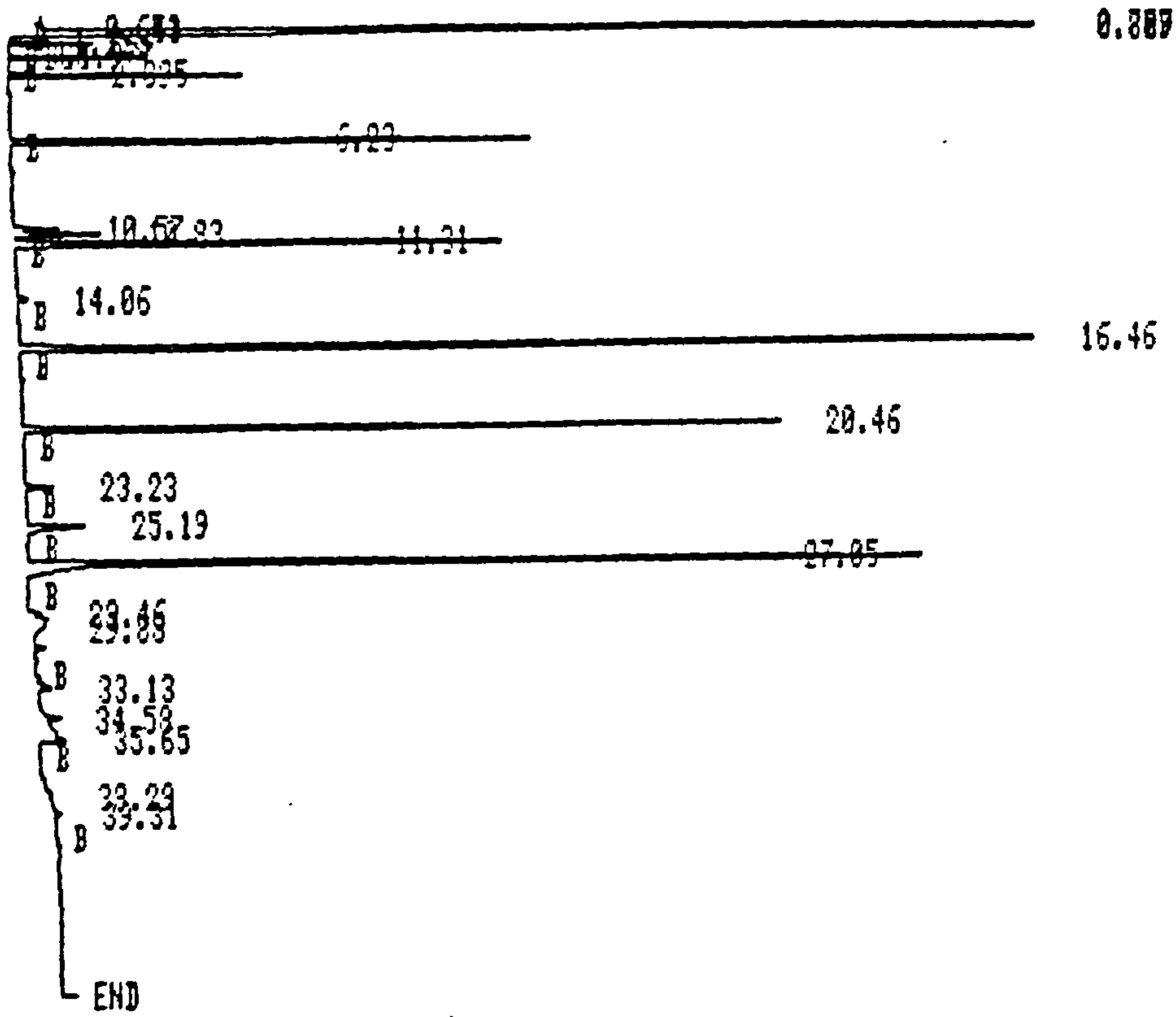
1) DB wax capillary column - a polar type

2) Carrier gas was Helium at 22.5 psi

3) Cold on-column injection

4) Set temperature programme (See Methods p53)

Figure 3.6.2a



It can be seen from the results that a total of seven sulphide compounds were identified, comprising approximately 60% of the total G.O composition. According to Lawson *et al.* (1990) detection by the GC method will identify approximately 60-80% of the total G.O. Yu & Wu (1989) stated that of the identified components, diallyl disulphide, methylallyl trisulphide and diallyl trisulphide constitute approximately 60% of the G.O composition, whereas analysis of the chromatograph indicated that these three sulphides comprise approximately 79% of the total sulphide composition.

3.6.3 Stability Of Stored G.O Samples Determined By GC Analysis

GC analysis of the sulphide composition of G.O samples stored in sealed glass vessels at -20°C were compared to samples stored at room temperature, to evaluate the long term stability of G.O. The results are presented in Table 3.6.3a.

Table 3.6.3a Sulphide Content Of Two Stored G.O Samples

Sulphide mg/g	G.O Stored at -20°C	G.O Stored at Room Temperature
Diallyl sulphide	9.4	9.6
Methylallyl disulphide	44.5	42.2
Diallyl disulphide	232.2	249.1
Methylallyl trisulphide	115.9	132.2
Diallyl trisulphide	188.3	218.5
Methyl trisulphide	9.7	9.6
TOTAL mg/g	600	661.2

From these results it can be seen that both samples indicated differences in terms of the individual sulphide content with respect to the "fresh" (stored at 4°C) G.O sample (Table 3.6.2b), although neither sample is statistically different with respect to sulphide composition. It can be seen that samples stored at -20°C remain constant with respect to total sulphide composition (600mg/g) compared with "fresh" G.O (601.11mg/g), whereas analysis of the G.O samples stored at room temperature indicated changes in the total sulphide composition (661.2mg/g). Overall it can be seen that the sulphides are not stable with respect to time dependent upon the storage conditions.

3.6.4 Qualitative Determination Of The Sulphides Present In G.O By HPLC

Repeated qualitative HPLC analysis of G.O was performed in order to validate the reproducibility of the retention times obtained for each individual sulphide component. A typical HPLC G.O trace is shown in Figure 3.6.4a. Identification of the sulphide peaks (Table 3.6.4b) was determined using gas chromatography mass spectroscopy (GCMS) previously performed at Humberside University.

Table 3.6.4b Sulphide Identification From HPLC Analysis

Retention Time	Sulphides
4.13	(DMD) Dimethyl disulphide
4.65	(DAS) Diallyl sulphide
4.98	(DMT) Dimethyl trisulphide
5.30	(DADS) Diallyl disulphide
5.68	(MATS) Methyl allyl trisulphide
6.59	(DATS) Diallyl trisulphide
7.07	(MATTS) Methyl allyl tetrasulphide
8.38	(DATTS) Diallyl tetrasulphide

A total of eight sulphides were separated by the HPLC method, of which two were identified as the higher sulphides methylallyl tetrasulphide (MATTS) and diallyl tetrasulphide (DATTS) not detected by GC analysis (by comparison of retention times to those obtained by Lawson *et al.*, 1991a). This appears to suggest that these higher sulphides (tetrasulphides) are relatively stable at the lower temperatures employed in the HPLC method and can therefore be identified. Methylallyl disulphide (MAD) which was identified by GC analysis, was not identified by HPLC analysis. According to Lawson *et al.* (1991a), the two peaks at retention times 9.23 and 11.10 are likely to be methylallyl pentasulphide (MAPS) and diallyl pentasulphide (DAPS) respectively.

Although direct quantification of the sulphides present in G.O cannot be achieved by HPLC analysis (due to the absence of internal standards), further work on the analysis of G.O (in terms of differences in individual sulphide peak areas) was performed using the HPLC method due to the limited availability of suitable GC methods at the University of Wolverhampton.

Figure 3.6.4a: HPLC Analysis Of G.O

10mg of G.O was extracted with 10ml of ACN, the extract was then filtered through an HPLC filter and 5µl of the extract injected directly into the HPLC.

Conditions Of HPLC:

1) ODS2 Column

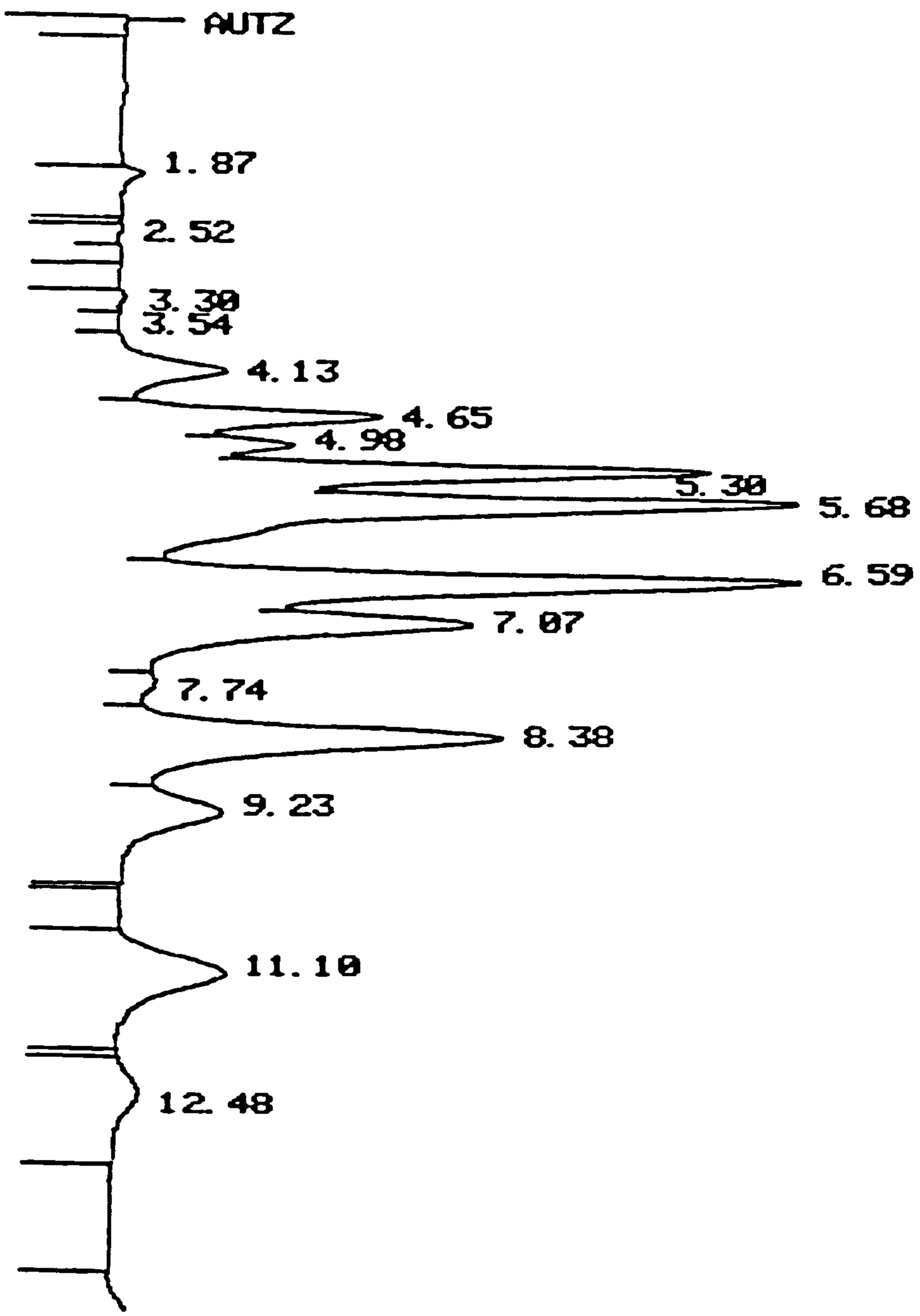
2) Mobile Phase = ACN:H_2O:THF (70:27:3)

3) Flow Rate = 1.0ml/min

4) UV detector absorbance at a wavelength of 254nm

Peak Identifications are given in **Table 3.6.4b.**

Figure 3.6.4a


IEEE NO
01
SAMPLE
S155
METHOD
METHOD1
TIME
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AUTZ
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4.13
4.65
4.98
5.30
5.68
6.59
7.07
7.74
8.38
9.23
11.10
12.48

3.6.5 HPLC Analysis Of The Effect Of Various Incubation Conditions On The Level Of G.O Sulphides Present In TSB

The aim of these experiments was to test the hypothesis that progressive volatilisation of G.O sulphides occurs, thereby diminishing observed antimicrobial activity (Section 3.2 p88), during incubation of G.O in aqueous environments.

The effect of temperature and agitation on the sulphide component levels in G.O within TSB (in tin foil capped boiling tubes and tightly screwed down screw-capped universal bottles, with no cap seal) over 24 hours were analysed using the HPLC system. The results (Figures 3.6.5a, b) are presented in terms of individual sulphide peak area over the 24 hour period of incubation for each of the components within G.O, compared to a control of fresh G.O.

The results indicated that the relative peak areas of all the eight sulphides decreased significantly after both static (4-100%) and agitated incubation (7-100%) of G.O for 24 hours at 37°C compared to the control of freshly prepared G.O (as shown in Table 3.6.5c).

Figure 3.6.5a: The Effect Of Various Incubation Conditions On The Sulphide Components In G.O (In Tin Foil Capped Boiling Tubes) By HPLC Analysis

Figure 3.6.5b: The Effect Of Various Incubation Conditions On The Sulphide Components In G.O (In Screw Capped Universal Bottles) By HPLC Analysis

2 x triplicate, 1.37mg/ml preparations of G.O in TSB (total volume of 10ml) were prepared in tin foil capped boiling tubes and air tight universal bottles. One set of tubes and bottles were placed under static conditions at 37°C in an incubator for 24 hours, the other set placed under agitated conditions at 37°C in a shaking water bath for 24 hours.

After incubation, 10ml ACN was added to each of the bottles, filtered by passing through a 0.2µm dynagard filter and 5µl of this was then injected into the HPLC system run under conditions stated in Methods Section 2.1.6.3.1 (p55)

A control of fresh G.O was analysed as a control.

LEGEND:

DMD DAS DMT DADS MATS DATS MATTS DATTS

Figure 3.6.5 a

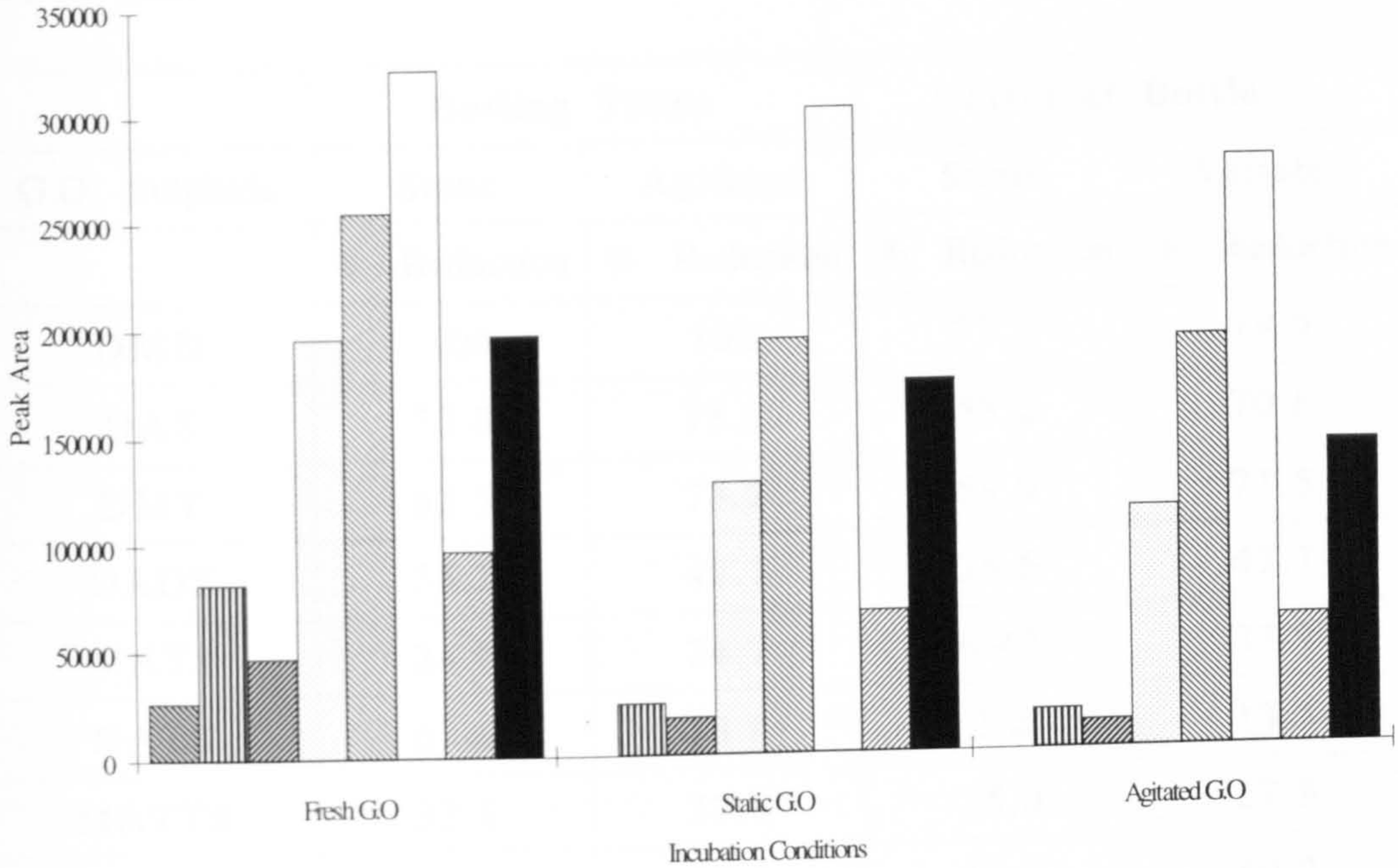


Figure 3.6.5 b

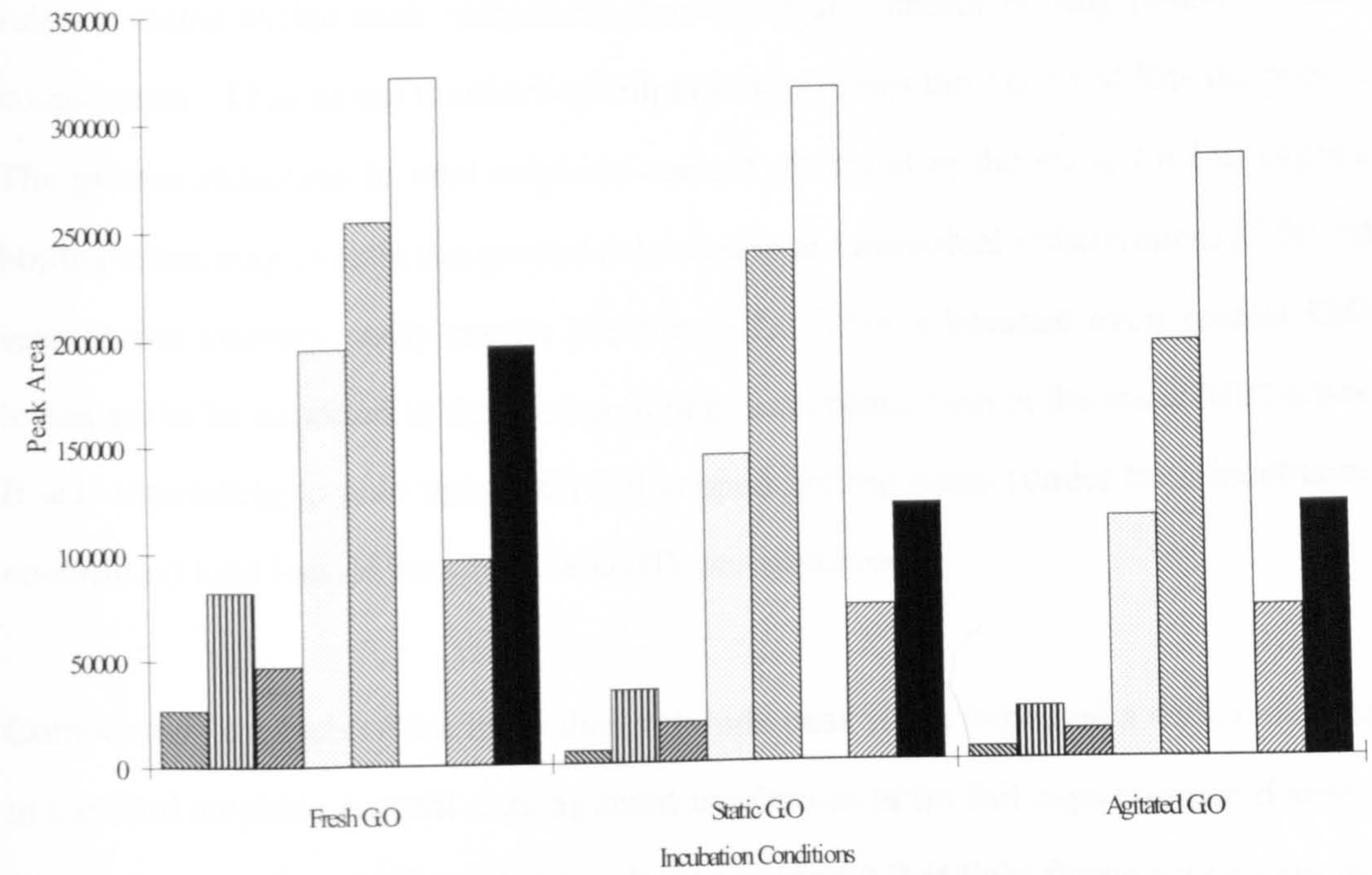


Table 3.6.5c % Reduction Of Each Sulphide Component

Note: The figures represent the % reduction in sulphide components compared to that of fresh G.O.

	Boiling Tubes		Universal Bottles	
G.O Sulphide	Static	Agitated	Static	Agitated
	% Reduction	% Reduction	% Reduction	% Reduction
DMD	100	100	77.7	79.5
DAS	70.8	78.2	58.2	70.6
DMT	62.5	73.2	59.9	71.5
DADS	35.2	42.7	26.6	42.7
MATS	24.0	24.7	6.47	23.9
DATS	6.06	13.8	2.2	13.5
MATTS	32.1	37.6	25.0	27.8
DATTS	11.95	27.5	38.8	40.2

From the results obtained it was observed that all sulphide components undergo reductions with respect to incubation status. With respect to the allyl and methyl containing sulphide components it appears that this loss is related to the number of sulphur atoms within each component, molecular size and/or boiling points of these components. Thus as the numbers of sulphur atoms rises the extent of loss decreases. The greater reduction in total sulphide content observed in the static tin foil capped boiling tubes may explain the greater reduction in antimicrobial effectiveness of the oil seen in the viability study results (Section 3.2). This is because even greater G.O losses are to be expected in those of sampling disturbance than in the static MIC tubes. It was interesting to note that in tin foil capped boiling tubes (under both incubation conditions) total loss of the sulphide DMD was observed.

Comparative analysis of the individual sulphide peak areas indicated a 46% reduction in the total sulphide content after agitated incubation in tin foil caps compared with a 16% reduction after static incubation. It was observed that tight fitting screw caps did

not prevent but did reduce the amount of volatilisation after incubation with (30%) and without agitation (12%).

The difference in % reduction between the two types of vessel used can be explained by; 1) within universal bottles the lower % reduction than in boiling tubes is probably due to the screw-caps although the rubber inserts were removed. Volatilisation of the sulphide components to the head space and past the cap threads will also occur and further loss of sulphide components occurs when the caps are removed; 2) within the tin foil capped tubes the higher % reduction is presumably due to loss in terms of volatilisation of sulphide components to the atmosphere above the liquid and external to the tube via the loose fitting tin foil. This was suggested also by the noticeable odour in the incubation cabinents during this experiment; 3) agitation of the G.O in TSB samples enhances the rate of volatilisation as a consequence of the increase in diffusion gradients between the oil/water/air interfaces.

3.6.6 Solubility Of G.O In TSB

The aim of this experiment was to determine the solubility of G.O within TSB in conjunction with MIC determinations in order to provide further insight into the MIC results obtained. The results of HPLC analysis of the top, middle and bottom fractions of a 10% suspension of G.O in TSB are presented in Figures 3.6.6a-c.

It can be seen that the top and middle fraction samples contain substantially lower concentrations of the G.O sulphide components (in terms of peak area) than that of the bottom sample, however by comparison of the individual sulphide peak areas to that of Figure 3.6.4a it was observed that all three fractions were greatly reduced with respect to individual sulphide concentration. The relative sulphide concentration of the top, middle and bottom fractions was approximately 4%, 7% and 46% respectively compared to 100% in "fresh" G.O (Figure 3.6.4a). In all three fractions, 7 of the total 8 sulphides detectable by HPLC were present whereas DMD was absent. These results therefore reflect the incomplete but significant solubility of G.O sulphides in aqueous

systems and a limiting rate of diffusion in the G.O+TSB system, which produces a steep concentration gradient between the bottom and top of the fluid column in the MIC tube.

Figure 3.6.6a: HPLC Analysis Of The Top Fraction Of A G.O/TSB Mixture

Figure 3.6.6b: HPLC Analysis Of The Middle Fraction Of A G.O/TSB Mixture

Figure 3.6.6c: HPLC Analysis Of The Bottom Fraction Of A G.O/TSB Mixture

4 x 10% preparations of G.O in TSB (total volume 10ml) were prepared in universal bottles and whirlymixed to produce a homogenous suspension. From 1 universal 10mg was immediately removed and extracted into 10ml ACN for HPLC analysis. The other bottles were allowed to stand for 20 minutes to allow the G.O to settle out. 10mg was then removed from the top, middle (interface between the TSB and G.O) and the bottom of the universal bottles and extracted into 10ml of ACN for HPLC analysis run under the conditions stated in Section 2.1.6.3.1.

Figure 3.6.6a
TOP

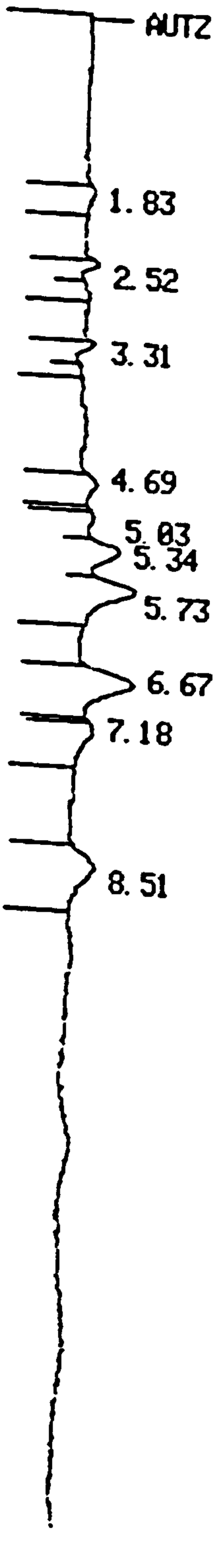


Figure 3.6.6b
MIDDLE

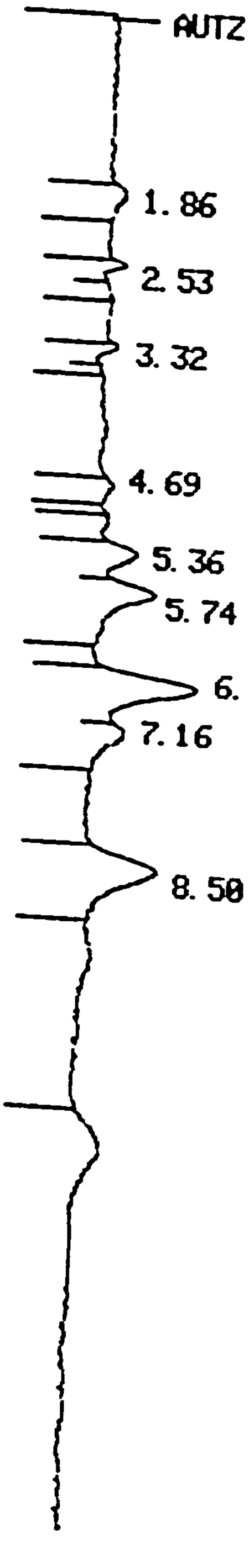
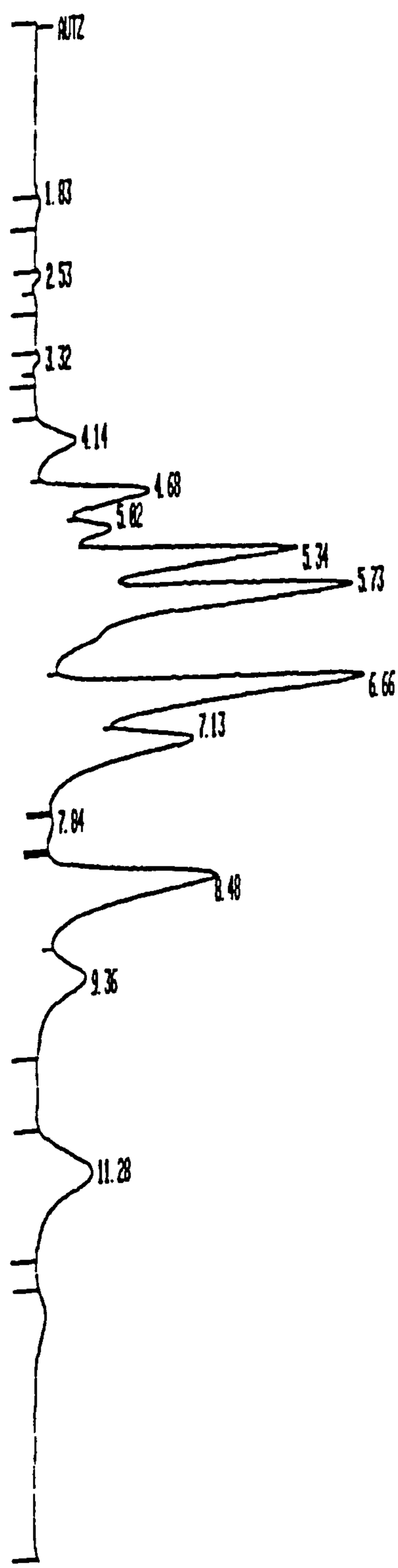


Figure 3.6.6c
BOTTOM



3.7 HPLC Analysis Of Changes In G.O Sulphide Composition During 24 Hour Incubation In The Presence Of Microbial Cells

The aim of this experiment was to monitor the event of a loss in individual sulphides present in G.O, with respect to time in the presence or absence of microbial cells. These studies could assist with the interpetation of both the MIC (Section 3.1) and viability study (Section 3.2) results with G.O treatment. They may also shed light upon the slight discrepencies between the effects of G.O observed in these studies.

HPLC analyses of G.O sulphides with respect to time was monitored so that changes in sulphide composition with respect to G.O in TSB in the presence and absence of microbial cells could be compared. The results of this experiment are presented in Figures 3.7a and b and revealed that during the 24 hour period of experimentation under static conditions in the absence of cells (Figure 3.7a) a reduction in the G.O sulphide components of 38% occurred. Comparison of this control experiment with the results obtained in Section 3.6.5 indicated similar findings.

In the presence of cells (Figure 3.7b), it can be seen that there is a decrease in the majority of the sulphide components (45%) with respect to time, which is not significantly (6%) higher than that of the control. However it should be noted that the sulphide MATS appeared to decrease and the sulphide DATTS appeared to increase at a much faster rate in the presence of cells than in the control. These differences appear to be related to the initial decline in *E. coli* cell population size. The results therefore suggest that there may be an interaction between the G.O sulphide components and the presence of bacterial cells. This could be of importance in understanding the nature of the antimicrobial effects of G.O. Also if the presence of bacteria affects the balance and quantities of G.O sulphides present it could influence the extent and nature of G.O's antimicrobial effects.

Figure 3.7a: HPLC Analysis Of Changes In Sulphide Composition Of A 1.37mg/ml G.O Concentration In TSB With Respect To Time

Figure 3.7b: HPLC Analysis Of Changes In Sulphide Composition Of A 1.37mg/ml G.O Concentration In TSB In The Presence Of *E. coli* (40) With Respect To Time

2 x Triplicate 1.37mg/ml dilutions of G.O in TSB were prepared in screw-capped sterile universal bottles to a total volume of 10ml. To the two sets of test triplicate bottles, 20µl of an overnight culture of *E. coli* (40) was added as an inoculum. The other set of bottles were used as controls (that is no inoculum added). The bottles were incubated at 37°C in a water bath for 24 hours. The sulphide content of both control and one set of test bottles was measured with respect to time by adding to them an equal volume of ACN (left to stand 2 mins) before filtering through a 0.2mm dynagard filter. To the other set of test bottles the contents were filtered prior to addition of ACN. 5ml was then injected into the HPLC for analysis. The mean results of the triplicate samples are plotted with respect to time.

LEGEND:

DMD DAS DMT DADS MATS
DATS MATTS DATTS E. coli

Figure 3.7a

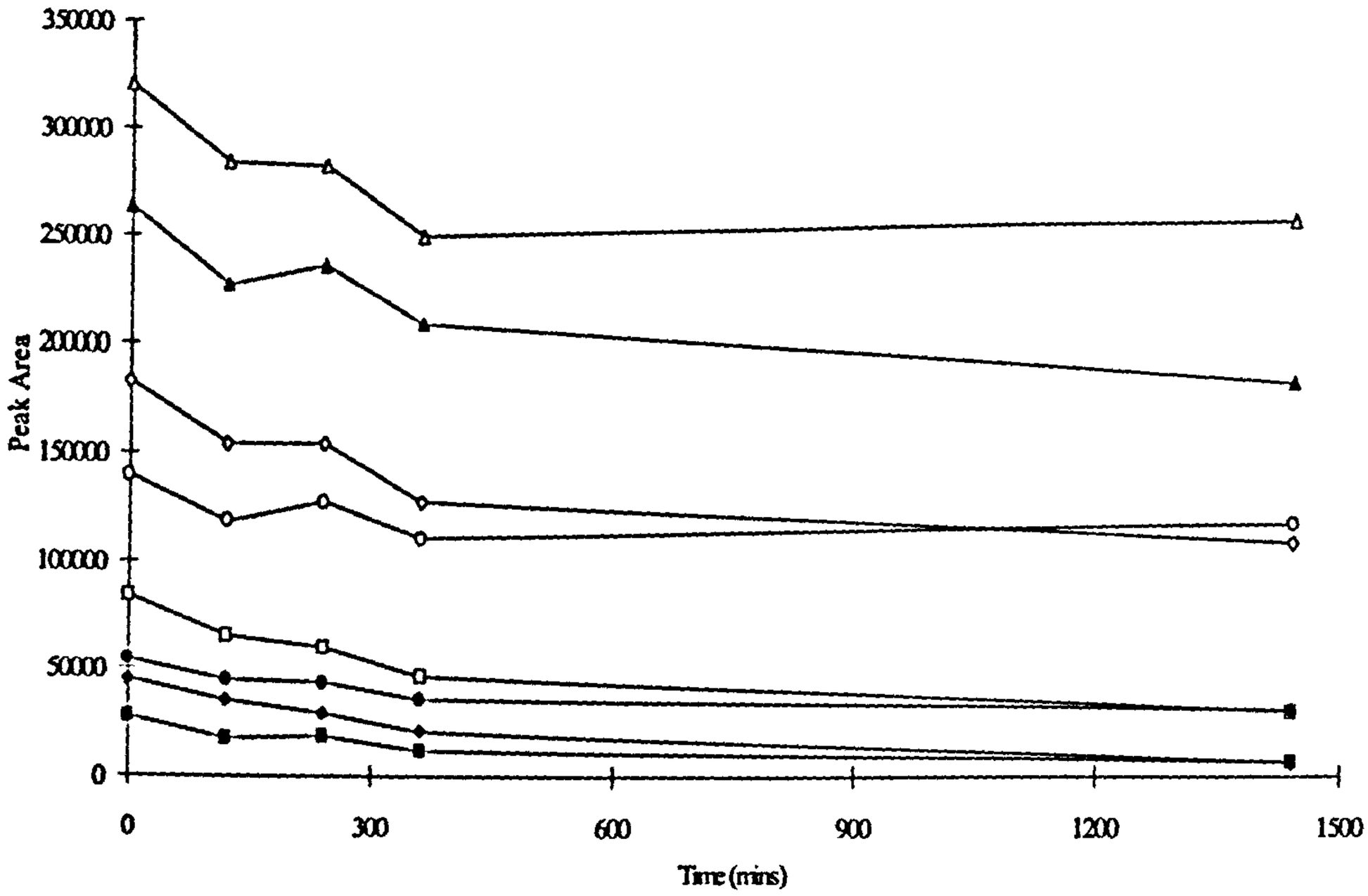
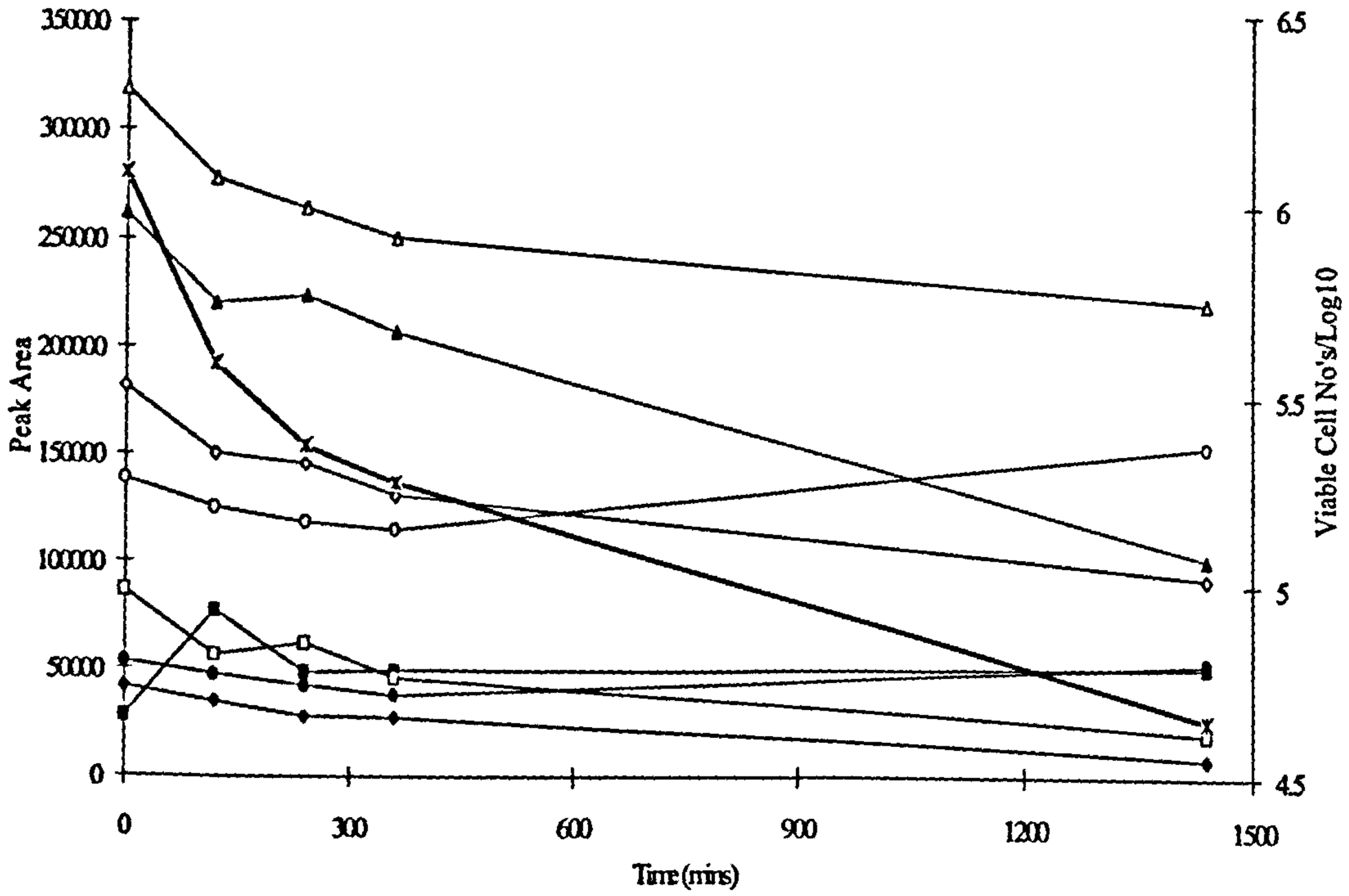


Figure 3.7b



SECTION 3 - The Effect Of Garlic Products On Enzyme Activity

3.8 Enzyme Studies

The aim of these enzyme studies was to gain a further insight into the modes of antimicrobial action of G.O, by investigating the effect of G.O and individual sulphide components (DADS and DMD) on the activity of two known sulphydryl enzymes; alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH). These enzymes are known to be inactivated by allicin-containing garlic preparations (Wills, 1956). It was therefore decided that a study of the effect of G.O and individual sulphides as well as that of allicin-forming G.P upon these enzymes would provide an interesting comparison.

The assay systems used to monitor the activity of each enzyme are described in the Methods Chapter (Section 2.1.7) along with the method for G.P allicin production (Section 2.1.1.1). The mean results (of triplicate enzyme studies) are presented in Figures 3.8a-d.

The results indicate that both the garlic products and the individual sulphides inhibited the enzymatic activity of both enzymes. Two-fold serial dilutions of both the garlic materials were associated with progressively lower levels of enzyme inhibition for both ADH (Figures 3.8a and b) and LDH (Figures 3.8c and d). Inhibition of ADH activity occurred immediately upon contact of the enzyme with the garlic product, such that 43% inhibition of ADH activity was achieved at the highest G.P concentration (100mg/ml), while at the highest G.O concentration (0.34mg/ml) 38% inhibition was achieved. For this particular enzyme further inhibition of activity by both garlic preparations occurred with respect to time. Thus, after 120 minutes incubation of ADH with G.P (at 100, 50 and 25mg/ml concentrations) complete (100%) inhibition of the enzyme was achieved, and for the highest G.O concentration (0.34mg/ml), 98% inhibition was achieved after 150 minutes.

In the case of LDH, it was observed that although inhibition of activity occurred immediately upon contact with the garlic products (approximately 50% and 44% inhibition with G.P (100mg/ml) and G.O (0.68mg/ml) respectively), the overall level of inhibition was generally constant with respect to time of incubation. Only in the cases of the two highest concentrations (Figure 3.8d, 100 and 50mg/ml) were slight increases in inhibition observed with time. It appeared that the level of inhibition was only slightly related to the concentration of G.P and the stable inhibition by G.P was the same irrespective of the G.P concentration in the ranges of 100 to 3.125mg/ml respectively. This was, however not the case for G.O, where slight increases in enzyme activity were observed with time, except in the case of the highest G.O concentration utilised, 0.68mg/ml. In addition reversal of inhibition of enzyme activity was observed after 50 minutes of incubation.

Figure 3.8a: The Effect Of Pre-Incubating Various Concentrations Of G.O With ADH On Its Enzymatic Activity.

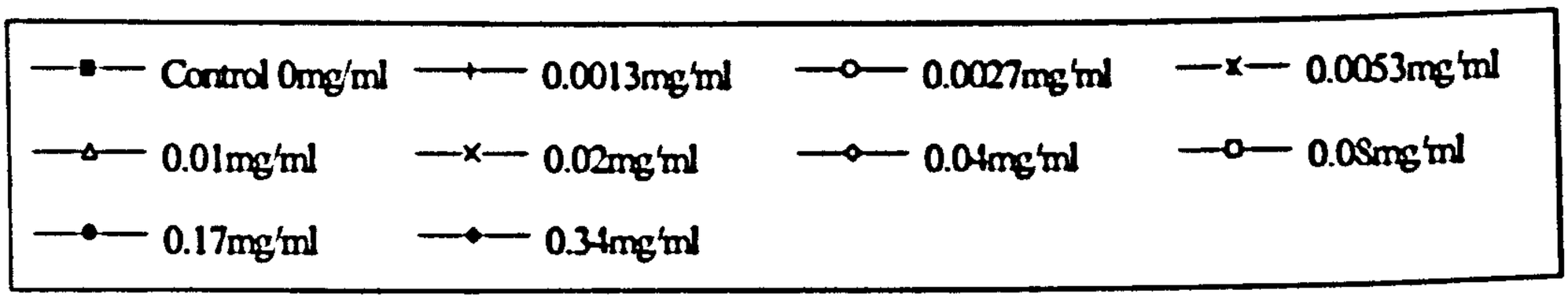
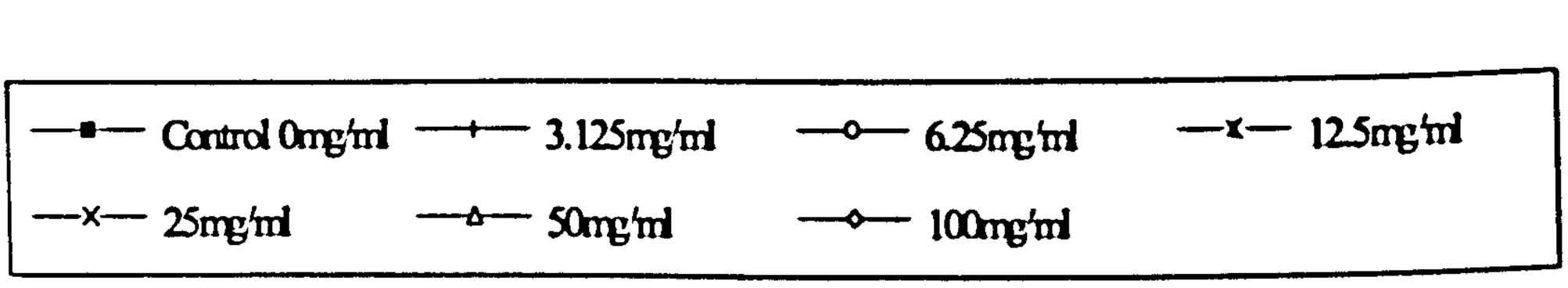


Figure 3.8b: The Effect Of Pre-Incubating Various Concentrations Of G.P With ADH On Its Enzymatic Activity.



Two-fold serial dilutions of both garlic products in double distilled water were prepared in screw capped bijoux bottles to a volume of 200µl. To each dilution, 200µl of ADH was added. The activity of the enzyme was measured (in terms of reduction of NAD^+) at regular time intervals using a PYE UNICAM 1800 SP Spectrophotometer linked to a PYE UNICAM detector. Results are expressed as enzyme activities after preincubation with G.O or G.P at the concentrations indicated.

Figure 3.8a

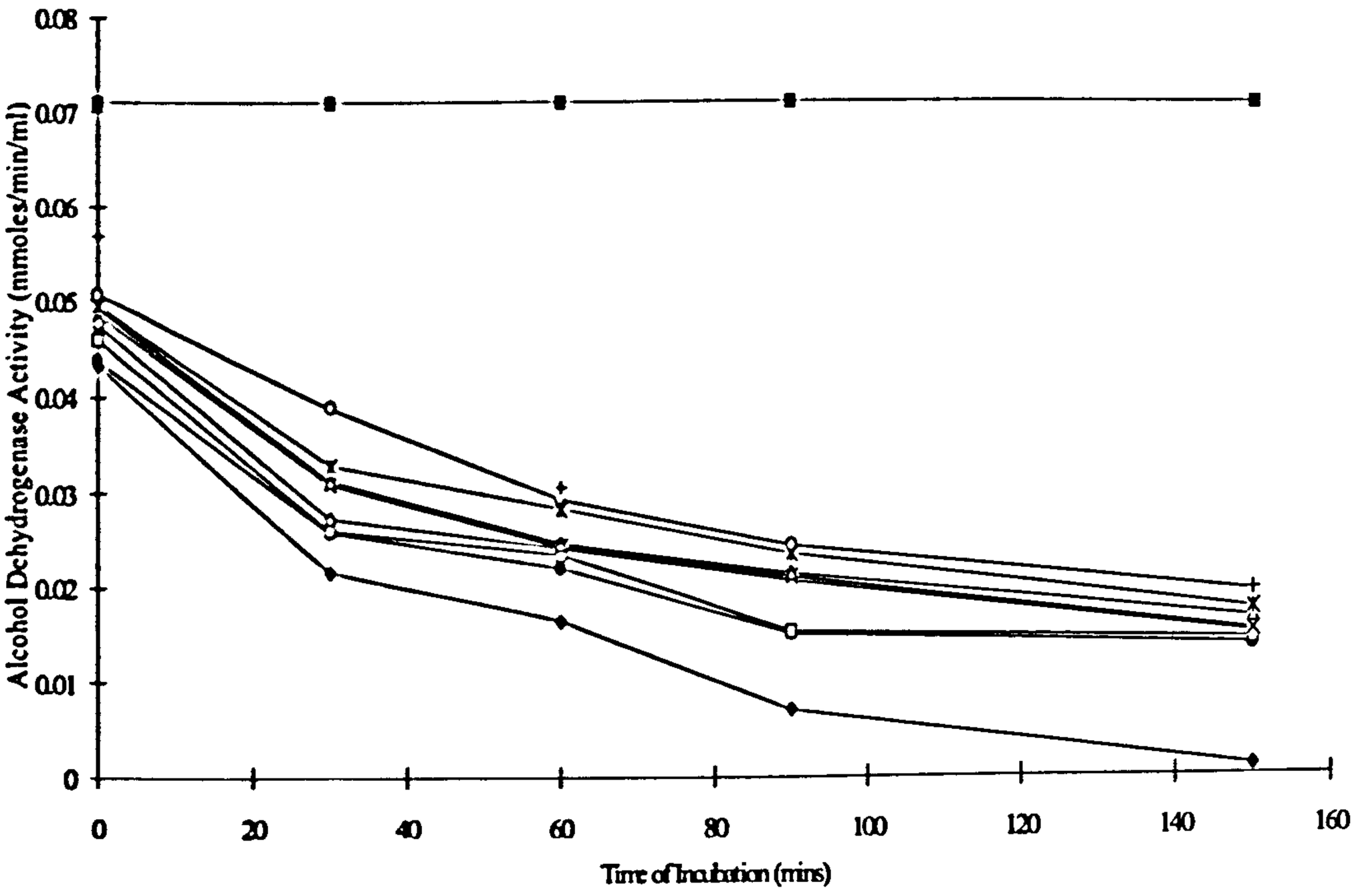


Figure 3.8b

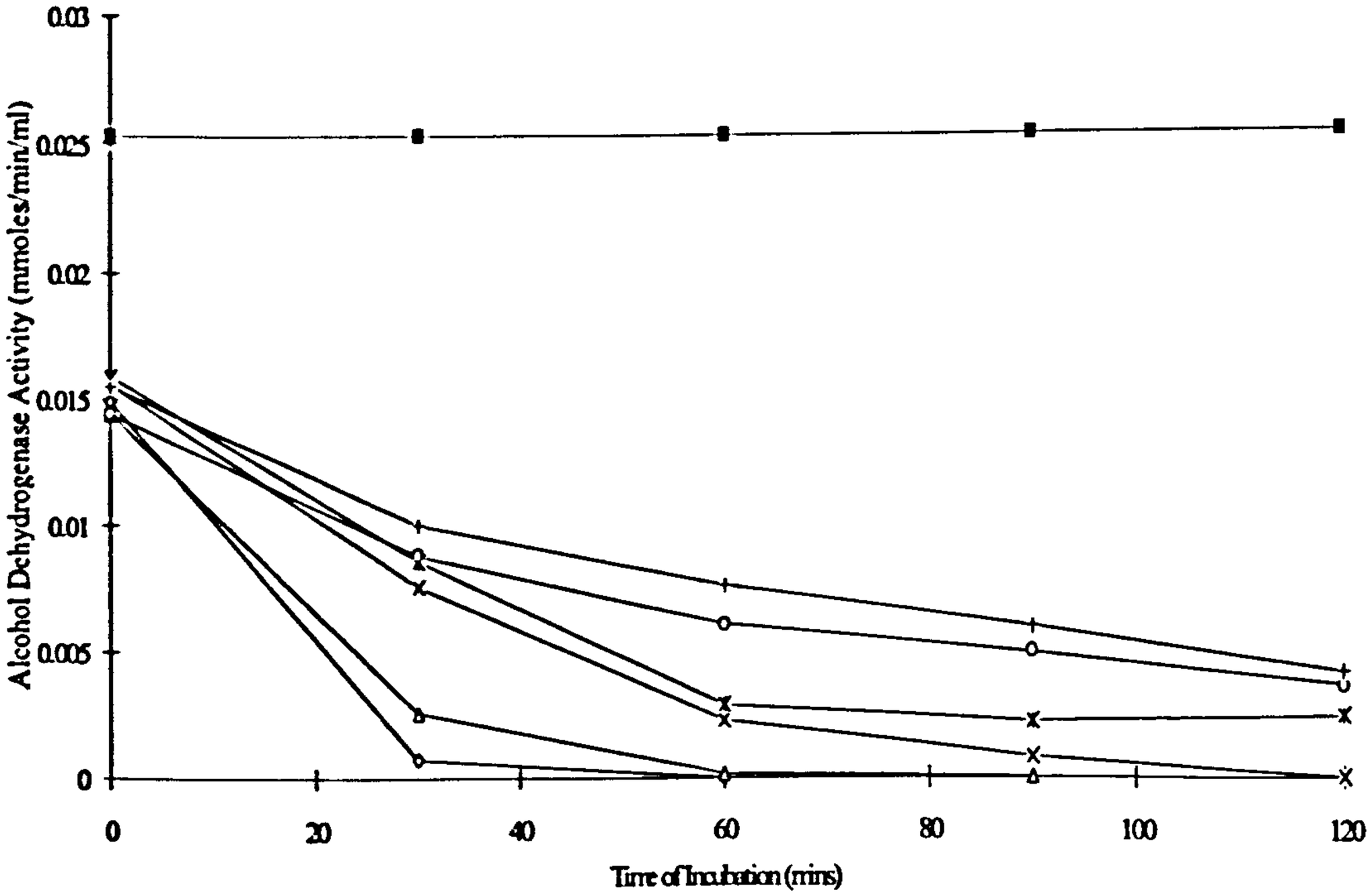


Figure 3.8c: The Effect Of Pre-Incubating Various Concentrations Of G.O With LDH On Its Enzymatic Activity.

LEGEND:

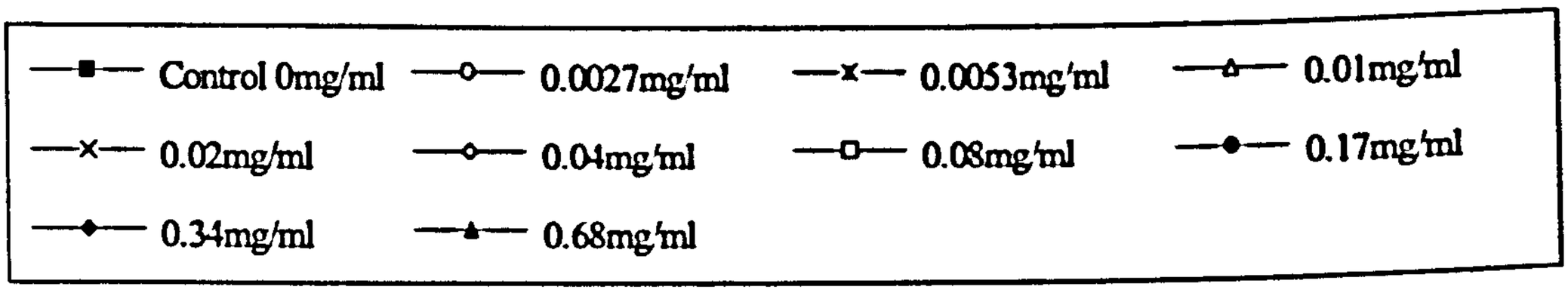
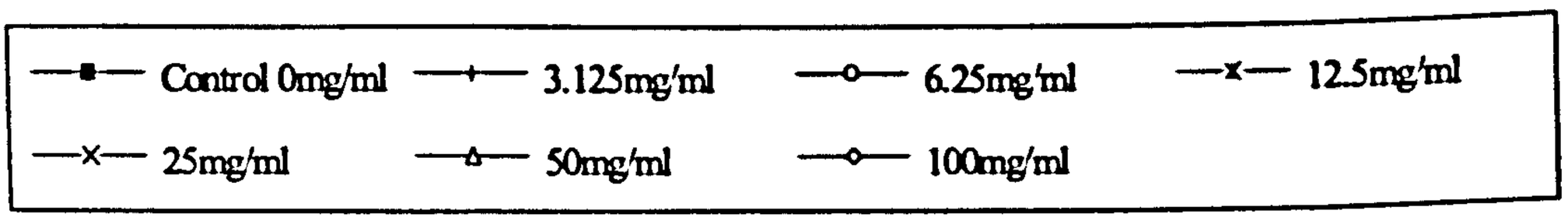


Figure 3.8d: The Effect Of Pre-Incubating Various Concentrations Of G.P With LDH On Its Enzymatic Activity.

LEGEND:



Two-fold serial dilutions of both garlic products in double distilled water were prepared in screw capped bijoux bottles to a volume of 200µl. To each dilution, 200µl of LDH was added. The activity of the enzyme was measured at regular time intervals using a PYE UNICAM 1800 SP Spectrophotometer linked to a PYE UNICAM detector. Results are expressed as enzyme activities after preincubation with G.O or G.P at the concentrations indicated.

Figure 3.8 c

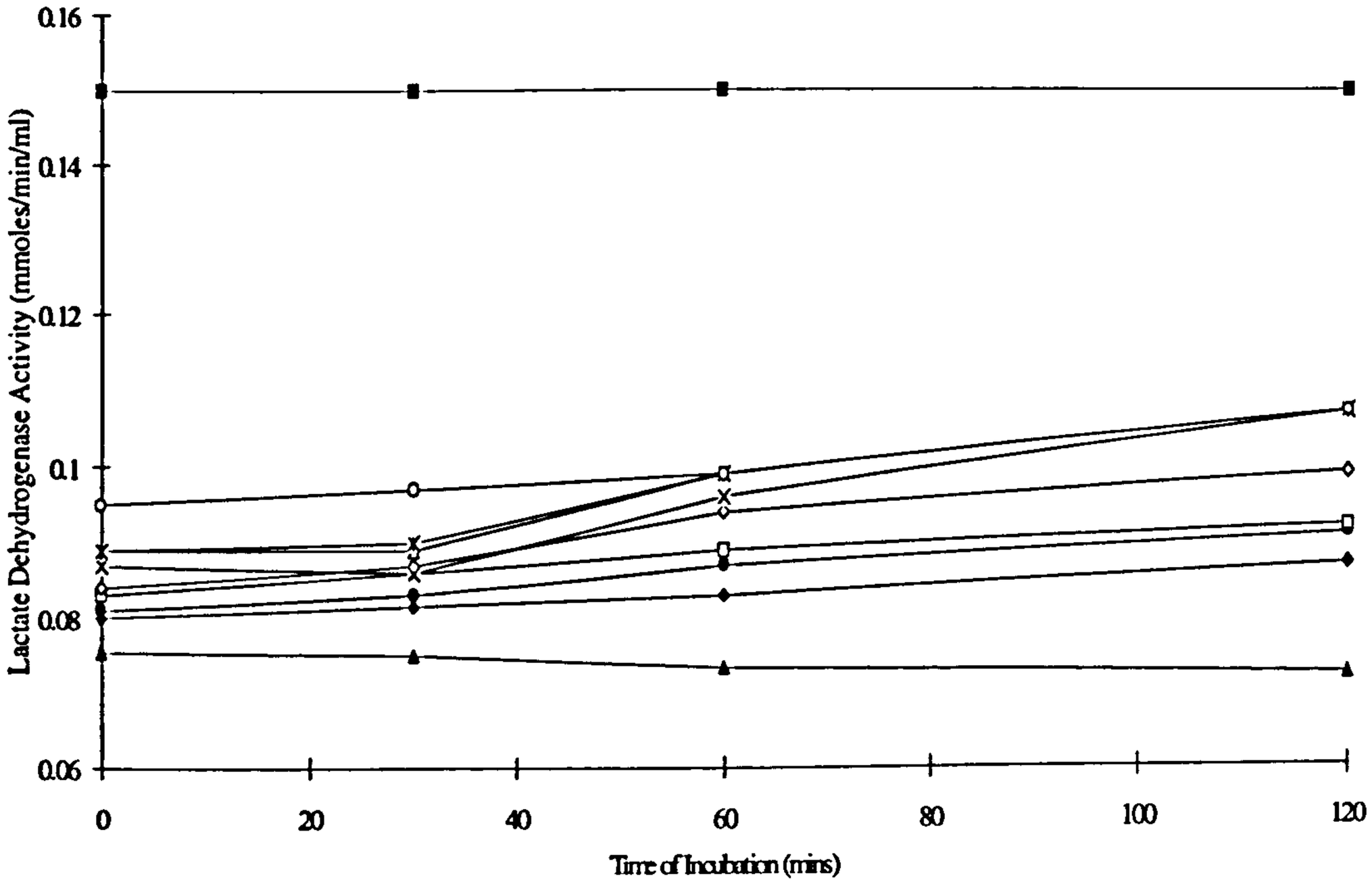
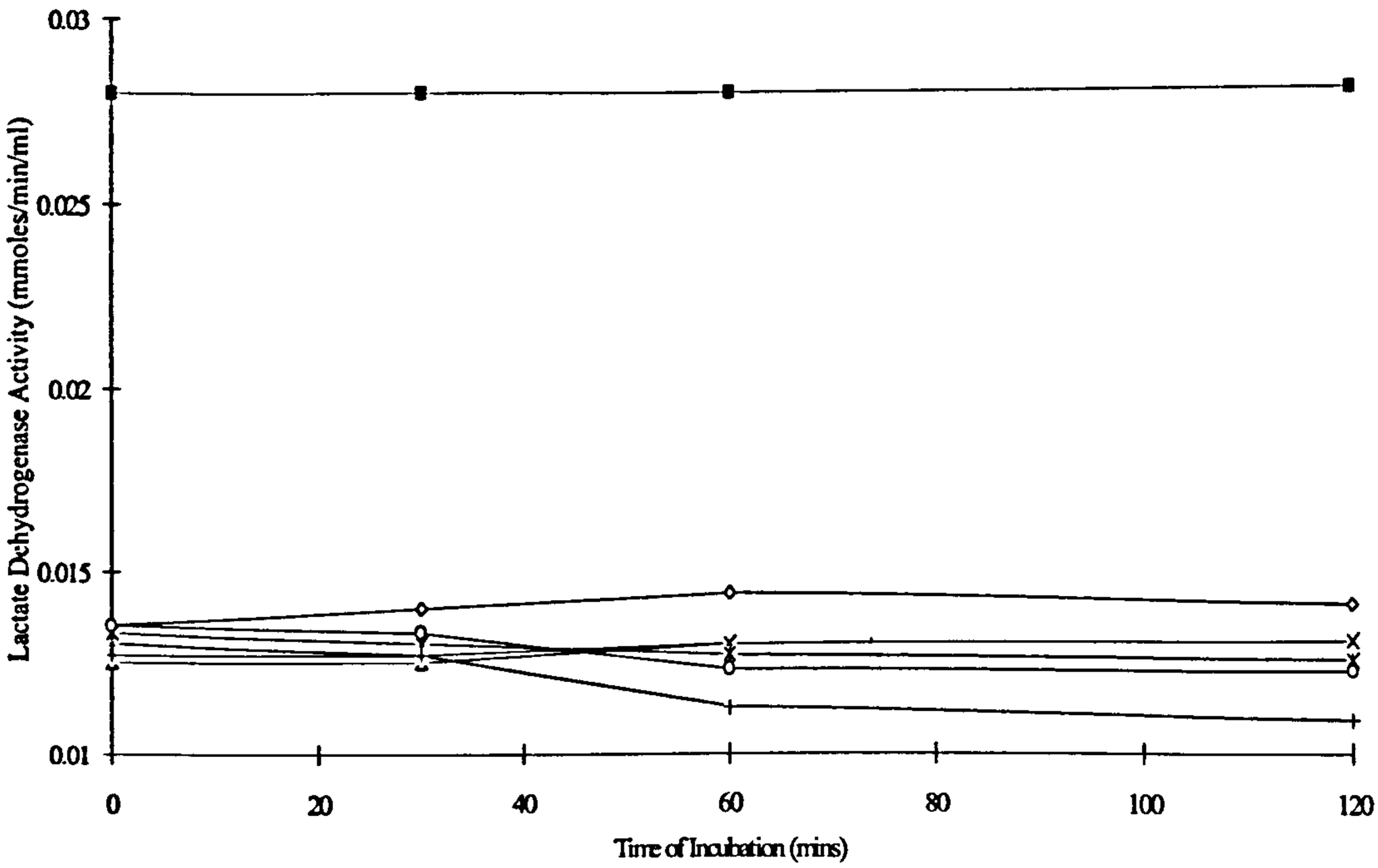


Figure 3.8 d



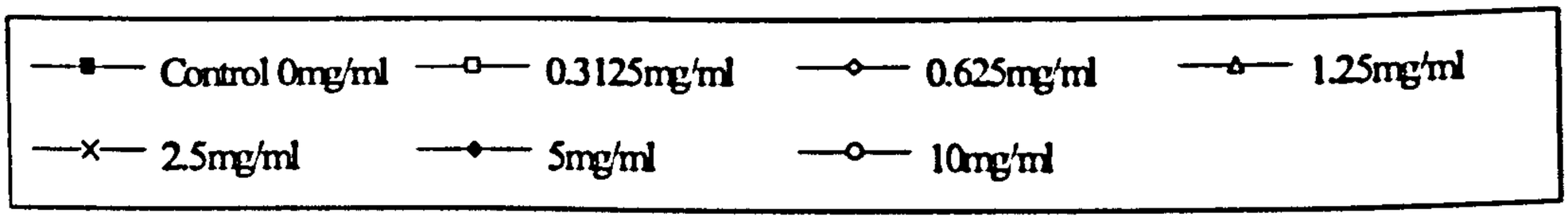
It was hypothesised that the inhibitory effect of G.O on ADH and LDH activity was a consequence of component sulphides in G.O. In order investigate this proposed activity of individual sulphides, the effect of two commercial sulphide preparations, DMD and DADS on the enzymatic activity of LDH were then performed. The results are presented in Figures 3.8e, f.

The effects of both DMD and DADS on LDH were similar to those observed with both G.O and G.P, in that they exhibited an immediate effect upon the activity of LDH (at t=0 60% and 53% inhibition for DMD and DADS respectively). In addition the inhibition of LDH activity by both sulphides was also shown to be stable over the time period of 120 minutes and appeared to be independent of the individual sulphide concentration in the range of 10-0.3125mg/ml. Thus although much higher sulphide than G.O concentrations were utilised the two lowest sulphide concentrations (Figures 3.8e and f, 0.625 and 0.3125mg/ml) gave very similar % inhibitions to the two lowest G.O concentrations (Figure 3.8c, 0.0027 and 0.0053mg/ml). This forms an interesting contrast to the higher antimicrobial potency apparently associated with G.O as compared to individual sulphides (Section 3.4).

Figure 3.8e: The Effect Of Pre-Incubating Various Concentrations Of DADS With LDH On Its Enzymatic Activity.

Figure 3.8f: The Effect Of Pre-Incubating Various Concentrations Of DMD With LDH On Its Enzymatic Activity.

LEGEND:



Two-fold serial dilutions of both commercial garlic sulphides in double distilled water were prepared in screw capped bijoux bottles to a volume of 200µl. To each dilution, 200µl of LDH was added. The activity of the enzyme was measured at regular time intervals using a PYE UNICAM 1800 SP Spectrophotometer linked to a PYE UNICAM detector. Results are expressed as enzyme activities after preincubation with DMD or DADS at the concentrations indicated.

Figure 3.8 e

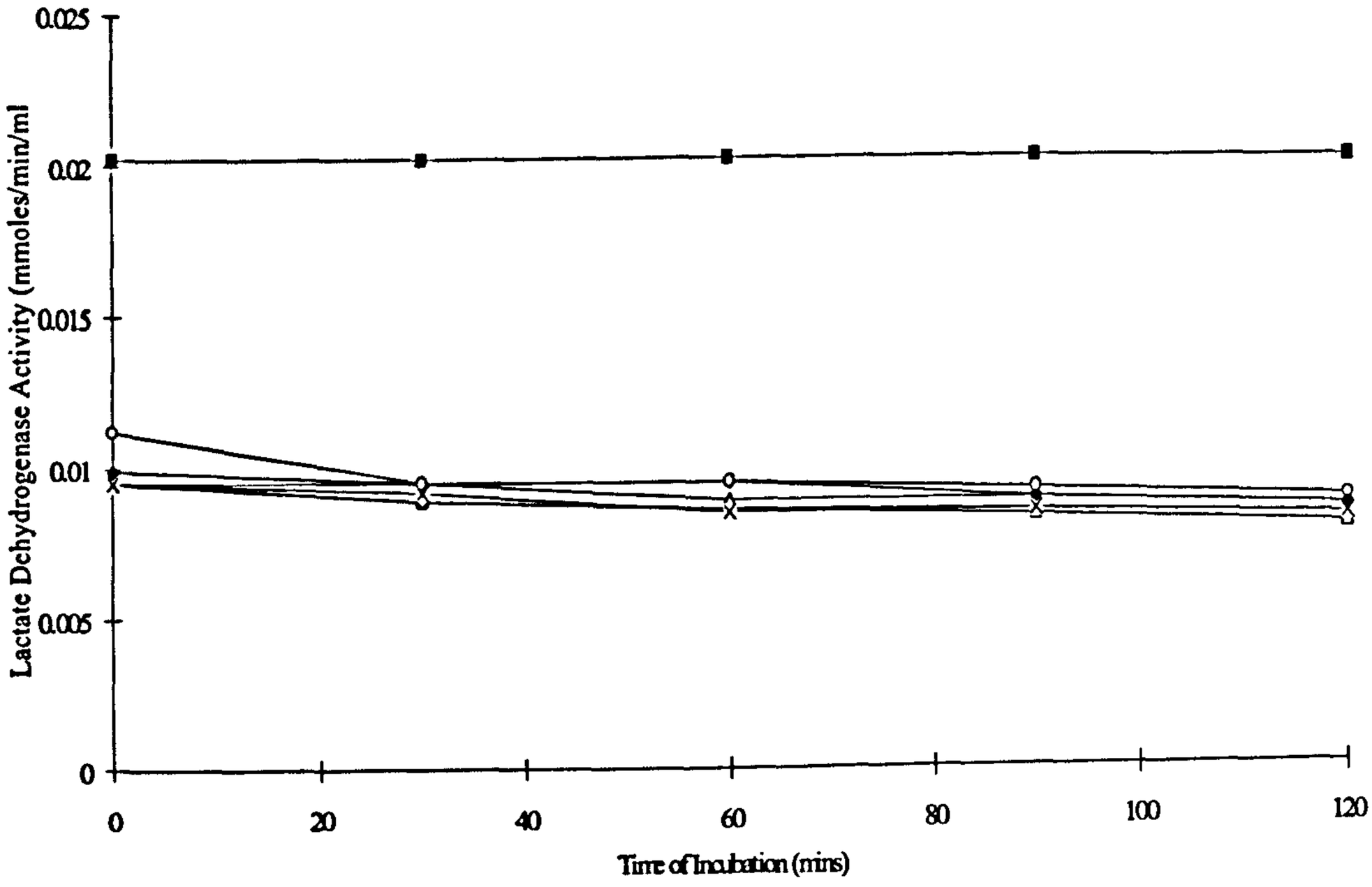
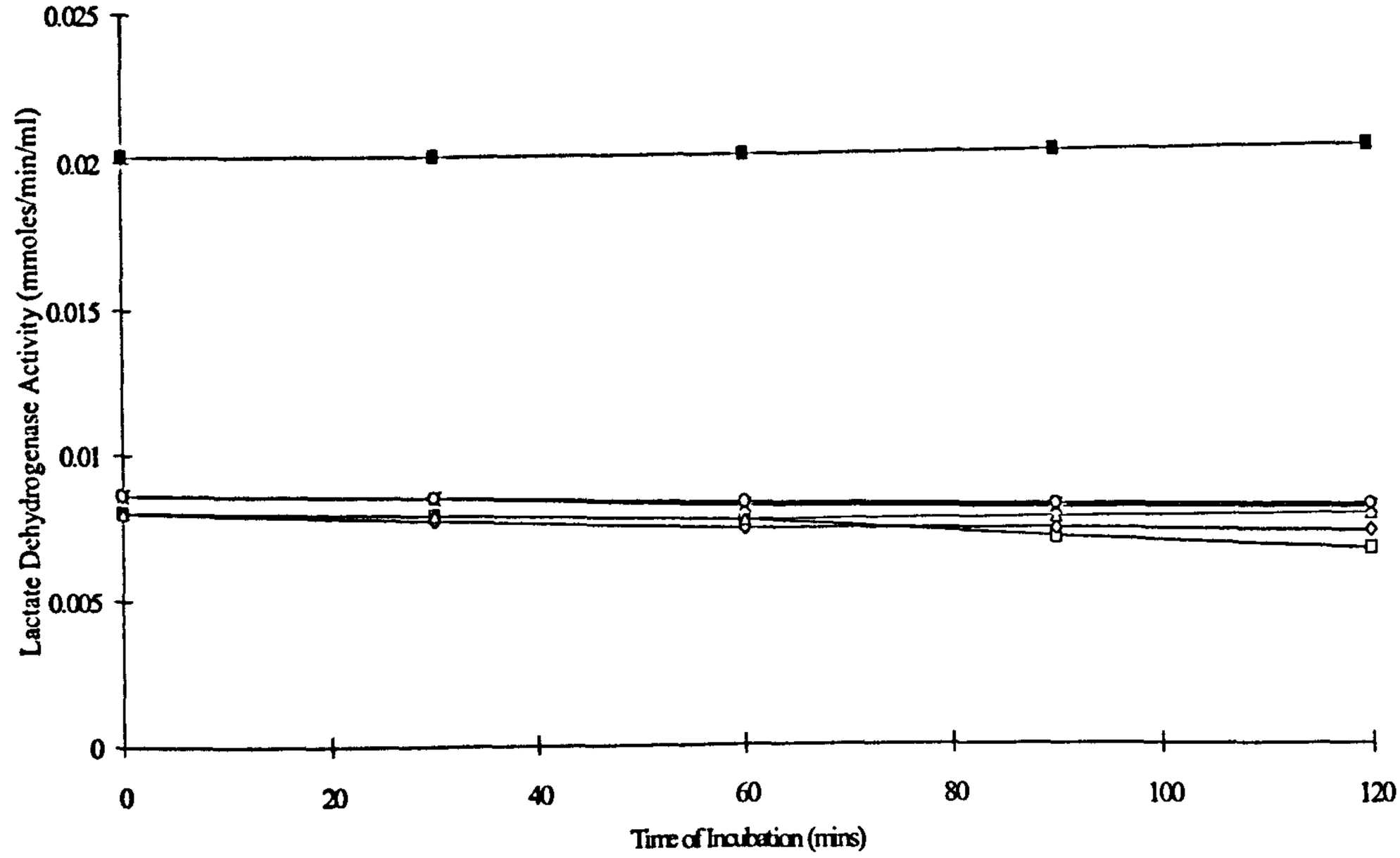


Figure 3.8 f



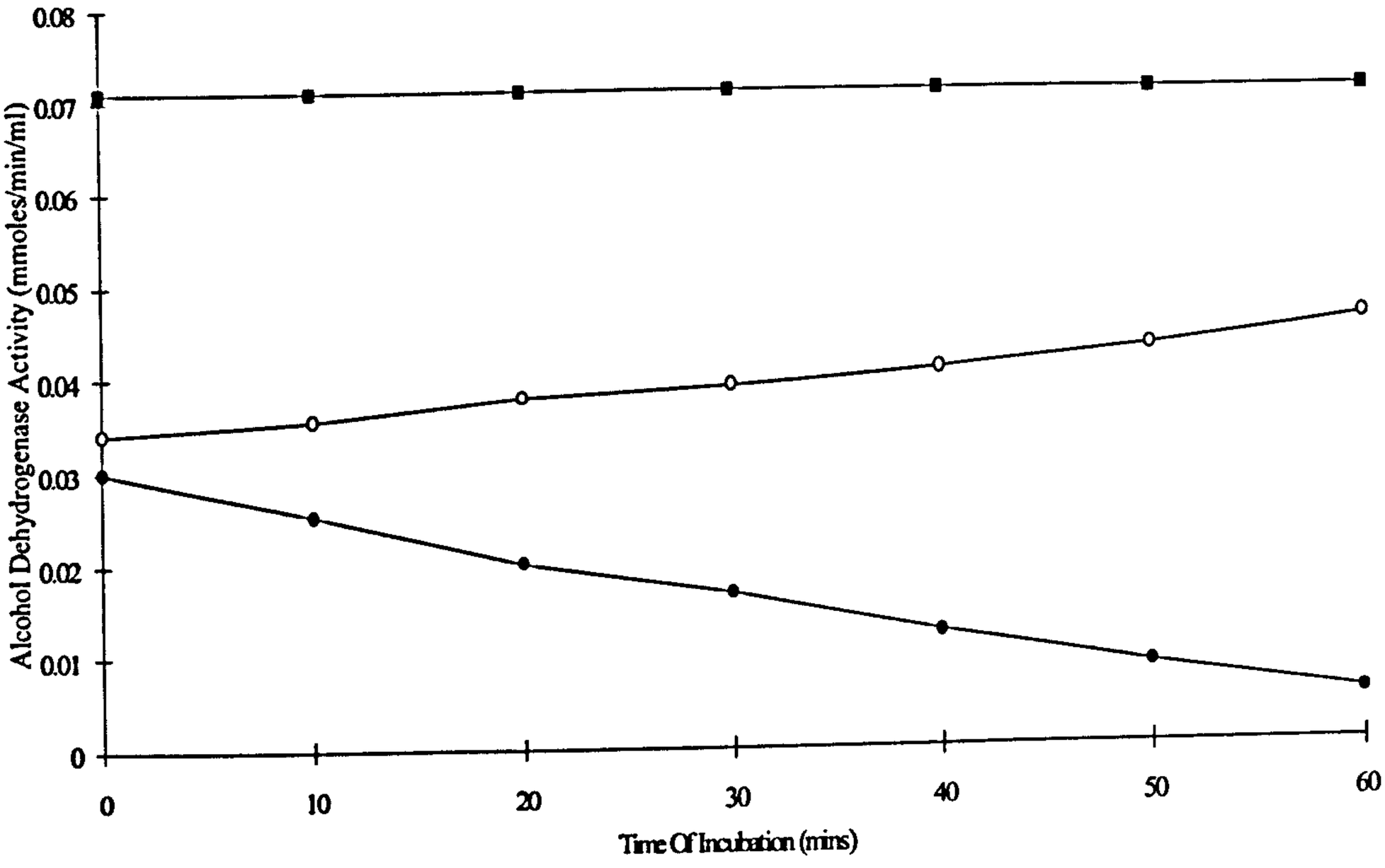
It was suggested by Wills (1956), that thiol compounds such as cysteine and glutathione can protect enzymes against the activity of allicin. The presence of glutathione at a concentration of 1mM, present in the assay system for ADH, did not appear to affect recovery of G.O/G.P induced inhibition of ADH activity. It was therefore decided to pre-incubate the ADH enzyme with glutathione (1mM) in order to attempt to protect the enzymatic activity prior to the addition of G.O. The results shown in Figure 3.8g indicate that pre-incubation of the enzyme with glutathione prior to addition of the G.O did not significantly protect the enzyme against the immediate inhibitory action of G.O, as the initial inhibition of enzyme activity (51% inhibition) was still observed. However further incubation of the enzyme+glutathione+G.O mixture, resulted in the activity of ADH being partially regained over the 60 minute period to a level of 65% of the original activity, whereas with enzyme+G.O only the ADH activity decreased to 2% of the original activity as was previously observed. The observation that ADH regains its activity in the presence of GSH may result from GSH reacting with the G.O sulphides rather than protecting the enzyme itself by competing with the G.O sulphides for the enzyme active -SH site.

Figure 3.8g: <u>The Effect Of Pre-Incubation Of ADH With Glutathione Prior To Addition Of 2.75mg/ml G.O.</u>

To 200µl of ADH (100 U/ml) 200µl of GSH (at a concentration of 1mM) was added in a screw capped bijoux bottle. The activity of the enzyme was measured at regular time intervals using a PYE UNICAM 1800 SP Spectrophotometer linked to a PYE UNICAM detector.

LEGEND:

Figure 3.8 g


Alcohol Dehydrogenase Activity (mmoles/min/ml)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
10
20
30
40
50
60
Time Of Incubation (mins)

CHAPTER 4

DISCUSSION

4.0 DISCUSSION

Published data has shown that garlic either in its natural (bulb/cloves) or processed form (such as garlic oil perles and garlic powder tablets) offers a mild safe option for the treatment of many ailments, G.O having a UK product license allowing for the claim of a herbal remedy for colds, coughs, rhinitis and catarrh (Fulder, 1990).

The escalating numbers of reported food poisoning cases in the UK and their associated cost to the society, together with an increase in public interest towards natural health care products has led to an interest in the potential anti-pathogenic value of various herbal medicines and especially garlic. The work presented in this thesis was performed to investigate the potential of garlic products as antimicrobial agents against foodborne pathogens within the human intestinal tract.

4.1 Chemical Analysis Of The Garlic Products

Allicin was first suggested to be the primary active antimicrobial component of garlic products by Cavallito & Bailey (1944). It has been shown that allicin is unstable, readily degrading via several pathways to form the secondary products of various sulphides (Brodnitz *et al.*, 1971; Block, 1985), which contribute to the characteristic flavour and odour of garlic. This instability of allicin has led to difficulty in both quantitative and qualitative analyses required for identifying antimicrobial components in garlic preparations, in addition to evaluating garlic preparations for commercial marketing purposes.

Several GC & HPLC methods have been reported for the analysis of alliin, allicin and corresponding sulphides (Jansen *et al.*, 1987; Ziegler & Sticher, 1989; Iberl *et al.*, 1990a, b; Lawson *et al.*, 1991a, c). Both GC and HPLC methods have achieved satisfactory separation of the G.O sulphide components (Lawson *et al.*, 1991b; Yan *et al.*, 1992, 1993) and of G.P thiosulphinates (Jansen *et al.*, 1987; Yu & Wu, 1989; Lawson *et al.* 1991b).

4.1.1 Qualitative/Quantitative Analysis Of G.P

Initial quantification of an aqueous G.P extract used in this study was performed by spectrophotometric determinations involving the indirect measurement of thiosulphinates (including allicin) from pyruvate and from ammonia, the by-products of the enzymatic conversion of alliin to allicin (Section 3.6.1.1). Both these assay systems produced a result of 11.00 (±1.5) mg allicin/g powder (Section 3.6.1) some of which is probably subsequently transformed into other thiosulphinates (Hörheimer *et al.*, 1968). In contrast, indirect quantification of allicin by conversion to vinyldithiins followed by GC analysis (Section 3.6.1.2) gave an allicin yield of 6.07 (±1.0) mg/g powder.

Garlic powder HPLC analysis reveals a mixture of thiosulphinates; diallyl, allyl methyl, methyl allyl, 1-propenyl allyl, allyl 1-propenyl, methyl 1-propenyl and dimethyl of which allicin (diallyl thiosulphinate) is the major component (60%) (Lawson *et al.* 1991a). Of the remaining components methyl allyl, allyl methyl and 1-propenyl thiosulphinates are the next most abundant (total of 30%) and it has been reported (Lawson & Hughes, 1992) that these are formed by reaction of preformed allicin with methane sulfenic acid. Lawson *et al.* (1991a) quotes the thiosulphinate content of fresh garlic to be 4.75-5.37mg/g which equates to a thiosulphinate concentration in freeze-dried garlic of 11.88-13.43mg/g assuming no losses during preparation. However losses during preparation due to instability of the thiosulphinates do occur and there is also considerable variation amongst garlic clove types (Lawson & Hughes, 1992). Of the thiosulphinates produced from garlic powder Lawson & Hughes (1992) identified that 7.52mg/g was allicin. Comparison of spectrophotometric results obtained in this study (Section 3.6.1.1) for thiosulphinate yield appear to agree with these results. However it appears that GC results obtained (Section 3.6.1.2) provide an underestimate of the allicin content. Thus, the results (calculated on the fresh garlic results quoted by Lawson *et al.* cited above) of this method of quantification appear to suggest that conversion of

allicin to vinyldithiins may not be directly compared to the other quantification methods which has been suggested by Lawson *et al.* (1991c). Iberl *et al.* (1990b) stated that only about 25% of allicin may be detectable as dithiins. Yan *et al.* (1993), suggested that the conversion of the two vinyldithiins from allicin is temperature dependent and the ratio of vinyldithiins components is a function of the temperature programme used in the GC analysis, therefore the low recovery of thiosulphinates may be the result of incomplete conversion. Although GC methodology has frequently been used for the analysis of the degradation products of allicin, the instability of the thiosulphinates combined with the relatively high temperatures employed in GC methods means they are unsuitable for the direct analysis of these components.

HPLC analysis of thiosulphinates can provide promising results. Since it operates at room temperature, it is unlikely to cause thermal changes in the compound during analysis, allowing this method to be mainly used for the quantitative and qualitative analysis of alliin and allicin (Jansen *et al.*, 1987; Iberl *et al.*, 1990; Lawson *et al.*, 1991b). However, due to the absence of external standards for HPLC analysis, thiosulphinate identification in this thesis was performed on a peak "fingerprint" basis (Section 3.6.1.3) by comparison of the relative retention times with those of pure thiosulphinates in published data (Jansen *et al.*, 1987; Iberl *et al.*, 1990b; Lawson *et al.*, 1991c). It was observed that a constant retention time for allicin of approximately 6.80 minutes was obtained and linearity of the HPLC method of analysis for allicin was tested by comparison of the peak area against the concentration of G.P (mg/ml).

4.1.2 Qualitative/Quantitative Analysis Of G.O

A variety of columns have been used for GC analysis of G.O sulphides, from polar to non-polar and packed to capillary columns (Yu *et al.*, 1989; Saito *et al.*, 1989). G.O, is a mixture of sulphur containing compounds and a high resolution for their

analysis is essential, therefore a polar DBwax column was used in this study as it has been reported to achieve both a high resolution and good baseline separation (Yan *et al*, 1992).

Results from initial GC analyses of G.O (Section 3.6.2), performed at Humberside University showed that a total of seven sulphides were resolved (DMD, DAS, MAD, DMT, DADS, MATS, DATS, for full names see Abbreviations, page iv) each given a positive identification by GCMS. Quantitative estimation of the sulphides present in G.O was made by introducing an internal standard (dipropyl disulphide). This sulphide was chosen as; 1) it has a different retention time compared to the sulphides present in G.O, 2) is of reasonable purity >99% AND 3) is rarely reported to be present in G.O. The weight content of the seven identified sulphide components within G.O was estimated using the internal standard (See Appendix 5). Of the seven sulphides, it was shown that the three major sulphides (DADS, MATS and DATS) contribute approximately 60% of the identified sulphide composition. This is comparable to the findings of Yu *et al.* (1989) and Lawson *et al.* (1991a). It should also be noted that no vinyldithiins, indicative of the presence of allicin in the sample prior to injection, were found in the G.O sample, confirming the observations of Lawson *et al.* (1991a).

HPLC has rarely been utilised to estimate the sulphide content of G.O (Lawson *et al*, 1991a), however the system developed and used at Humberside University achieved high resolution and separation of the sulphide components. Ten sulphides were resolved, eight of which were given positive identification (Section 3.6.4). Four higher sulphides (MATTS, DATTS, MAPS and DAPS) resolved were not detected by the GC method. It was also noted that methylallyl disulphide (MAD), detected by GC was not identified on the HPLC trace. These differences are interpreted as resulting from the higher sulphides being unstable and subject to degradation to lower sulphides at the elevated temperatures employed by the GC

method. Thus although capillary GC has frequently been used for G.O sulphide analysis, no sulphides with more than three sulphur atoms have been reported using a polar capillary column (Vernin *et al.*, 1986; Yu *et al.*, 1989) and more than four sulphur atoms for heated diallyl disulphide (DADS), due to the instability of these compounds at the elevated temperatures required by GC (Block *et al.*, 1988; Iberl *et al.*, 1990a, b; Lawson *et al.*, 1991a, b). In contrast the HPLC technique used clearly showed the presence of tetra and pentasulphides so it can be stated that HPLC has an advantage over GC, as an ambient temperature method for analysing the thermally unstable G.O compounds.

4.1.3 Evaluation of GLC & HPLC Methods For Analysis Of G.O & G.P

The differences in quantification analysis of the garlic products observed using GLC and HPLC methods are probably due to the sensitivity of both methods for the garlic product components. HPLC utilises a UV detector, the signal intensity of which is proportional to the absorbance of the individual component in the sample, which in turn is proportional to the concentration of the components (Beer Lambert Law). However with UV detectors the intensity varies according to the type of molecule, whereas with the GC FID, molecules of all types yield the same signal intensity per molecule. Also, because of the difference in the detection system, HPLC has a relatively lower sensitivity compared with capillary GLC (Yan, Personal Communication). It was shown that between 20-120mg/ml G.P and 0.2-1mg/ml G.O, linearity of the HPLC detection (in terms of the respective allicin and individual sulphide peak areas) was high, while at low concentrations of 6.12-12.5mg/ml G.P and 0.0625-0.2mg/ml G.O it was not, presumably due to background "noise". A loss of linearity also occurred with higher concentrations of G.P and G.O (≥120mg/ml and 1.5mg/ml respectively) which may indicate that the extraction of allicin or sulphides at these higher concentrations is less effective than with the intermediate concentrations. Alternatively the poor separation of the components at these higher concentrations could explain the observed deviation

from a linear absorbance response. HPLC quantification can be achieved by prior calibration with pure sulphide components of the oil or thiosulphinates of the powder, in order to determine their extinction coefficients (Lawson *et al.*, 1991a, c). HPLC quantification of thiosulphinates or sulphides used in this study was not performed as no pure internal standards were available. To summarise, there are advantages and disadvantages in using either system for analysing garlic components. The limited GC facilities available and the thermal instability of garlic sulphides derivable from G.O led to the use of HPLC throughout most of this study. This method was used to provide comparative information on the total and individual G.O sulphide content of microbial media containing G.O.

4.2 Delivery Of Garlic Products To The Microbial Milieu

Although the antimicrobial activities of fresh garlic have been recorded since ancient times, comparative studies of the antimicrobial activity of the garlic products within the intestinal tract are few. In order to assess the sensitivity of a range of pathogenic and non-pathogenic microorganisms to the antimicrobial activity of both steam distilled G.O and reconstituted freeze-dried G.P, initial studies were performed by MIC determinations and selected viability studies.

The antimicrobial investigations performed with G.O and G.P attempted to ensure that as far as practicable; 1) the incorporation of the G.O and G.P into the liquid growth medium should facilitate the direct contact of the garlic product under investigation with the microorganisms tested AND 2) the antimicrobial components were present at constant levels with respect to the time-period of investigation.

In relation to facilitation of direct contact (that is 1 above), initial observations indicated that G.O was virtually immiscible in aqueous systems (such as TSB), tending to settle out of solution, forming globules. It was therefore hypothesised that the total antimicrobial activity of G.O may not be exerted in aqueous systems.

In order to try to produce a homogeneous mixture of G.O in TSB, Maslin & Hill (1990) investigated the effect of detergent addition on the emulsification of G.O. The results obtained indicated that by using appropriate levels of detergent all or most of the G.O could be dispersed and by using oil colorants (such as Sudan dyes) this was confirmed. Further experiments also showed that these detergent levels in the absence of G.O had no antimicrobial effect. However, when equal concentrations of G.O in the absence of the effective level of detergent were tested for antimicrobial activity, no differences were detected. It was therefore concluded that there was no purpose in using detergent to assist G.O dispersal within the aqueous phase to facilitate expression of its antimicrobial potential. In this study the solubility of G.O in aqueous environments similar to those used in the MIC/ viable count experiments performed was tested by HPLC analyses of different fractions obtained from a 10% G.O preparation in TSB (Section 3.6.6). Results indicated that the antimicrobial components of G.O (namely the sulphides) were present in the greatest quantity within the lower fractions (displaying the same MIC value as the original "homogenous" sample, p79). Considerably smaller quantities of the sulphides were present in both the middle and upper fractions (shown in the HPLC data), which also expressed no antimicrobial activity. These results suggest that the bacteriostatic/bacteriocidal concentration of G.O components are achieved by a process of continual solubilisation and removal of these components into the aqueous phase, then into the microorganisms. Alternatively, the hydrophobic G.O may transfer progressively to the lipid phase of the microorganism, possibly assisted by lipid components suspended in the predominant aqueous phase of the medium. This is supported by HPLC data showing the reduction of G.O constituents in the medium when cells are present (Section 3.7).

Cavallito *et al.* (1944); Johnson & Vaughn (1969); Petricic *et al.* (1978) assessed the antimicrobial activity of G.O using aqueous extracts of G.O or plate diffusion assays which depend, respectively, upon the solubility of G.O in water and mobility

of G.O within the aqueous phase of a solidified agar plate. These methods, unlike those of this study, would not have allowed significant exposure to either aqueous or hydrophobic phases of the bacteria tested and this probably explains the negative results obtained for G.O by these authors.

In relation to maintaining the constancy of antimicrobial components, experiments comparing MIC determinations performed on open or closed, agitated or static, G.O/TSB mixture were performed. The greatest MIC values occurred following exposure to and especially when agitated. These reductions in the antimicrobial activity of the G.O were attributable to volatilisation of an essential G.O component. This was confirmed by HPLC analyses (Section 3.6.5) which showed that a 46% reduction in individual G.O sulphide content occurred after agitated incubation with loose fitting caps compared to a 16% decrease after static incubation. It was further shown that tight fitting caps reduced the losses associated with agitation and static incubation to 16 and 4% respectively. These substantial losses in total sulphides by volatilisation cannot fully account for the greater G.O concentrations required in MIC/time-dependent studies (Section 3.2), if total garlic sulphides alone are considered. However it was shown that not all the sulphides present in G.O volatilise at the same rate. Thus qualitative HPLC analysis of the changes in G.O composition in TSB with respect to time (Figure 3.7a) indicated a decrease (in terms of peak area) of all the individual sulphides present in G.O over the 24 hour period of experimentation in the absence of cells. The decrease in individual sulphide concentrations ranged from 15-85%, with a 33% total loss in sulphide concentration. Different sulphides present in G.O volatilise at different rates dependent on their molecular size and boiling points. Thus it was found that the smaller molecular weight sulphides DMT, DMD and DAS exhibited reductions in concentration of 85%, 75% and 63% respectively over a 24 hour incubation period of G.O in TSB (tin foil capped). If these components were shown to be responsible for much of the antimicrobial activity of G.O then the observed losses in these

compounds due to volatilisation could account for the observed time-dependent losses in the antimicrobial properties of G.O-containing media with time. It was not feasible to test this possibility fully as part of this project, although differences in antimicrobial activity were observed (Table 3.4.1.1a; Figures 3.4.1.2a, b). Results suggested that DADS, DAS, DMD and DMT do not fully account for the antimicrobial activity of G.O, however the MIC results showed that DMT has a stronger antimicrobial action than G.O itself and the other individual G.O components, but this particular sulphide is lost more readily than the other components (Figure 3.7b).

An alternative possible explanation for the reduction in G.O effectiveness with respect to time within antimicrobial media is that the organisms concerned become progressively resistant to it. This was tested by daily subculture of *E. aerogenes* cells exposed continually to sub-lethal concentrations of G.O. A constant MIC value of 0.68mg/ml over 112.9 generations of an average cell population size ranging between 10^6-10^8cells/ml was found indicating no development of resistance to G.O. These results confirm the findings of Fulder (1990), that no garlic extract resistant bacteria have as yet been isolated. This result also appears to rule out another possibility, which is that garlic may induce the development of a slower growing sub-population of cells which tolerate G.O better than the main population and which show up as significant components later in time.

Another potential explanation of the evidence for a time-dependent decline in G.O effectiveness could be an interaction between the G.O sulphides and the bacterial cells. This could take the form of reduction of the total amount of G.O present. This was tested by HPLC analyses for G.O composition in conjunction with a cell population viability study (Section 3.7). Under these conditions a 37.7% total loss of sulphides was observed which was non-significantly higher than the control (33% in the absence of cells). This does not support the hypothesis that the

bacteria present remove G.O from the medium by metabolic action. Small changes in composition of two of the sulphides were however observed. But apart from those changes in MATTS (only a 3.1% reduction) and DATTS (only a 10% increase) all the allyl sulphides showed a substantially enhanced decrease in concentration (31-79%) measured as chromatograph peak areas as compared to the control (absence of cells).

Another possible source of time-dependent G.O activity loss is that the cell may associate with the antimicrobial components rendering them unavailable for further antimicrobial activity. Association could occur by; 1) specific adsorption of G.O droplets by the bacterial cell wall and consequently penetration by the G.O through the cell membrane into the cytoplasm, followed by reaction with cellular constituents. By binding with proteins and enzymes this could render the G.O droplets unavailable for further antimicrobial action. The properties of adsorption and penetration depend on the chemical nature of the G.O and the number of binding sites (if any) on the bacterial cell wall. Hill & Maslin (unpublished observations) indicated that there was evidence that high cell densities of cultures (>10^{10}cells/ml) require higher G.O concentrations to inhibit growth than cultures of lower cell densities (>10^{7}cells/ml), suggesting an absorption effect of the G.O reducing the availability of G.O in the environment OR 2) binding of the bacterial cells to the surface of the G.O droplets preventing diffusion of the G.O into the surrounding environment. In the presence of bacterial cells, reduced sulphide detection by HPLC analysis of filtered samples was observed. However after ACN addition, a large increase in sulphide content in the filtrate was seen (results not presented) suggesting a possible association between the G.O and bacterial cells.

A further possible cause of a reduction in G.O antimicrobial activity with time could be a time-dependent interaction with the growth medium constituents or as a consequence of changes in the chemical composition of the medium resulting from

the growth and activities of the bacteria therein. Although this could not be investigated directly, comparison of the effects of growth versus non-growth media and of the addition of various chemicals to the media (Section 3.3.3) to assess their influence upon G.O action indicated that alterations in salts concentrations might be influential, but that active growth of bacteria was not.

Cysteine additions to Minimal Salts Media (MSM) +G.O resulted eventually in "neutralisation" of the antimicrobial effects of G.O. This presumably results from cleavage of the garlic sulphides by hydrolysation and subsequent formation of sulphides with cysteine. It is inferred that a similar process occurs with tryptone in TSB (Section 3.3.3). This reaction could explain the slight (16%) reduction of sulphides observed in experiments in which G.O sulphides were measured following static incubation with TSB (Section 3.6.5). This small reduction is considerably (2-3x's) increased by agitation presumably due to greater volatilisation. The HPLC data (Section 3.6.5) indicated that G.O is not likely to be reduced by more than factors of 2-4 fold by either volatilisation or chemical reaction. The extents of reduction in G.O sulphides does not correspond to the greater (that is 15-20x's) increases in G.O MIC values in TSB reported in both static and agitated TSB media (3.3.1). Not tested directly but this lack of correspondance could be explained by increased/improved contact (resulting from agitation) and thence increased chemical reaction between SH-residues of the TSB and G.O sulphides.

From a practical point of view it was concluded from the experiments described that agitation of a G.O/TSB mixture would be undesirable as it would promote G.O sulphide loss and therefore reduce the antimicrobial effectiveness of G.O. Indeed the results of this study show that even where maximum practical precautions were taken to avoid volatilisation of G.O sulphides, a substantial loss (37%, Section 3.7) over 24 hours. The same (or similar) precautions, of tight sealing and no agitation, were taken against G.O loss in both MIC and viability studies

during growth experiments in this study. Therefore similar losses of around 37% of G.O are likely to have occurred in these experiments. In comparison with the sensitivity of MIC tests, which is generally taken to be one dilution (a factor of 2) this is a fairly small amount. The range of MIC values (Table 3.1d) with G.O was 1000-fold, also the reproducibility of the results obtained for particular strains are always within the range of 0.34-5.5mg/ml for *E.coli* (18 isolates) and 0.34-2.75mg/ml for *Staph. aureus* (5 isolates). Because of the need to take samples it was not feasible to restrict access of the media of growth experiments to the air. This inevitably led to additional loss of G.O from the medium (See p215). Sampling from growth media was therefore performed in as rapid and reproducible a manner as possible. The results obtained generally show highly consistent concentration-dependent effects (Figures 3.2a, c; 3.5.1c, 3.5.1.1b, d, f). In addition, where the same organism was tested on more than one occasion results did not differ by more than the error associated with viable counting procedures (error of 15-20% either way).

It has to be recognised that in performing experiments with a volatile material such as G.O it is not practicable to obtain completely reliable absolute values for antimicrobial activity. In comparative experimental terms, as was the case in this study, where replication was performed and reproducibility demonstrated this is not of great importance. Moreover in terms of assessing in a general way, the clinical potential of G.O, absolute amounts and concentrations established by *in vitro* studies are of limited predictive value. Not only are the effective doses liable to be affected by the complex physiology of the human host, loss due to volatilisation via the skin and intestinal tract occur in host situations as well. Similar arguments with respect to the validity and comparability of the results obtained apply to the possibility that certain G.O components which may have specifically high antimicrobial activities are especially rapidly lost (See p216).

Garlic powder products vary considerably in their content of alliin and therefore in their thiosulphinate-forming potential. This is mainly a result of the differences in the alliin content of garlic bulbs resulting from differences in strain and country of origin, although soil conditions may be of predominant importance (Lawson *et al*, 1991a). In addition the method and care used in processing the bulb are important (Lawson *et al*, 1991c). For these reasons, and so as to ensure a high and standardised level of activity, a G.P was selected that had been prepared by the best available freeze-drying procedure, and which had been proven to have an easily and reliably detectable level of antimicrobial activity in earlier studies at Wolverhampton (Hill & Maslin, 1991). This G.P had been obtained as a large single batch from the manufacturer (Interprise Ltd) and immediately stored in small aliquots under dry deep-frozen conditions to provide a high and standardised level of alliin and alliinase.

In contrast to G.O, aqueous extracts of G.P were visibly miscible within the variety of growth media assessed. This was expected since it is well established that thiosulphinates, such as allicin, are readily water soluble. However after 24 hours incubation of G.P extracts in TSB it was noted that at high concentrations (200-100mg/ml) a precipitate always formed. Analysis of this precipitate showed it to consist of proteinaceous material, presumably as a consequence of denaturation of plant proteins.

The stability of garlic thiosulphinates has been studied by Brodnitz *et al* (1971), who showed that the thiosulphinates of garlic are unstable and upon mild heating or even at room temperature. They stated that this non-enzymatic rearrangement of allicin leads to the formation of symmetrical and mixed mono-, di-, and trisulphides, as well as sulphur dioxide, of which the allyl and methyl sulphides are the main components in steam-distilled G.O. Work performed at Humberside University has shown that allicin completely disappeared within 30 minutes of

heating at 100°C, changing to various sulphides (methylallyl disulphide, diallyl disulphide and methylallyl trisulphide being the major three). Similar results were found by Yu & Wu (1989), who worked on the stabilities of allicin in garlic juice. Thus in aqueous systems the garlic thiosulphinates, such as allicin, which are formed when G.P dissolves in water are converted to their corresponding sulphides (at 40°C for 144 hours). This is also their fate in animal systems (Egen-Schwind *et al*, 1991). HPLC analysis of G.P pre-incubated at 37°C for 24 hours using the G.O/HPLC conditions utilised in this study, did not indicate the presence of any sulphide components, probably due to the very mild conditions investigated. It is therefore tentatively concluded that during the 24-48 hour time period of the MIC and viability experiments performed within this study, little or no conversion of the thiosulphinates to their sulphides occurred. What is less certain is the extent to which thiosulphinates may have been lost from the medium by volatilisation, however it is known that the thiosulphinates have a lower volatility than sulphides (Lawson *et al*, 1991a), suggesting that any loss due to volatilisation may be minor.

In summary, the availability, stability and retention in solution of G.P thiosulphinates were probably high during the time period and under the conditions of the experiments performed. However some slight loss of antimicrobial activity is likely and taken into account in explaining the results.

4.2.1 Antimicrobial Activity of G.P

The MIC results obtained indicated a narrow band of values (3.125-25mg/ml) covering all species and strains of bacteria tested, suggesting a limited variation in sensitivity of the different species of microorganisms towards the G.P. Each of the MIC determinations were performed in triplicate and the final value always fell within one dilution (the expected limits of error for this method). The range of MIC values obtained for *E. coli* strains (3.125-12.5mg/ml) were similar for all bacteria tested. This is surprising because it shows that the strains of a single

bacterial species, which are presumably associated with relatively minor intraspecific differences in bacterial structure or physiology, are associated with a similar range of antimicrobial sensitivity to the very wide range of bacterial species tested. These results suggest that the antimicrobial action of G.P may be very unselective. The sensitivity range obtained in this study was similar to that described by Hughes & Lawson (1991) for garlic clove homogenates (7.5-15mg/ml) against *E. coli* (ATCC 25923). Rees *et al.* (1993), using a G.P preparation against the same species of organism but different strains indicated a lower but again limited MIC range (0.6-2.5mg/ml).

The MIC results also indicated that Gram +ve and Gram -ve bacteria display a similar susceptibility to the G.P. Thus three Gram +ve species (including *Bacillus spp, L. acidophilus* and *L. monocytogenes*) showed an MIC range of 6.25-25mg/ml, whilst eight Gram -ve species showed an MIC range of 3.125-25mg/ml. Moreover viability studies of the Gram +ve species *L. monocytogenes* (433a) and *Bacillus spp* (17) (results not presented), showed that inhibition of *L. monocytogenes* and *Bacillus* growth occurred at 3.125mg/ml and <3.125mg/ml G.P concentration respectively and that a 25mg/ml G.P concentration was bactericidal after only 90 minutes incubation. Viability studies of the Gram -ve species *E. aerogenes* (3) and *E. coli* (427) showed a similar pattern of results. The similarity between the sensitivities of Gram +ve and -ve bacteria to G.P adds supports to the idea that the antimicrobial action of G.P is rather non-specific (as mentioned above). In contrast, these findings do not support the findings of Small *et al.* (1947), that there is a relationship between Gram cell wall reaction and garlic susceptibility nor therefore their linked suggestion that differences in bacterial cell wall permeability to allicin exist which depend upon these cell wall differences. It should be noted, that the hypothesis of Small *et al.* (1947) was based upon screening only *Staphylococcus spp, Streptococcus spp* and *Bacillus spp* as examples of Gram +ve bacteria and no examples of Gram -ve bacteria were tested. In contrast comparisons made in this thesis involved

determinations on a wider range (Table 3.1c) of both Gram +ve and -ve bacteria (3 and 8 respectively). It is not ruled out that cell wall composition may affect susceptibility since such an effect may be masked by other physiological cell attributes such as extracellular cell wall coatings like lipopolysaccharide.

MIC tubes sampled and the viability counted at 24 and 48 hours showed that cell death, not merely growth inhibition, had occurred in a number of cases. This was especially so at concentrations well above the MIC value (results not shown). A more detailed study of both inhibitory and bacteriocidal effects was achieved by time-studies of cell population growth, showing that G.P in the growth medium exerted a concentration-dependent effect on the various phases of *E. aerogenes* cell population growth and death (Figures 3.2c, d). Thus at lethal G.P concentrations (>3.125mg/ml), the rate and level of cell population death was garlic concentration-dependent and at sub-lethal G.P concentrations (<1.56mg/ml), the length of the lag phase of growth and cell growth rate were garlic concentration-dependent. Similar results indicative of a bactericidal effect at appropriate G.P concentrations were also found with *L. monocytogenes* (433a), *Bacillus spp* (17), *E. coli* (427) (results not presented).

These observed effects on bacterial growth and viability are comparable to published results observed with other bacterial species in the presence of fresh garlic (Subrahmanyan *et al*, 1957, 1958; Abdou *et al*, 1972; Dankert *et al*, 1979; Shashikanth *et al*, 1984), dried garlic (Johnson & Vaughn, 1969), garlic extracts (Tynecka & Gos 1973; Karaioannoglou, 1977; Mantis *et al*, 1978; DeWit *et al*, 1979; Kumar & Sharma, 1982; Elmina *et al*, 1983; Dabaneh & Al-Delaimy, 1984; Kumar & Gupta, 1984; Chowdhury *et al*, 1991; Rees *et al*, 1993) and pure allicin (Cavallito & Bailey, 1944; Hughes & Lawson, 1991).

4.2.2 Antimicrobial Activity Of G.O

MIC determinations of G.O were performed in triplicate against a wide range of species and strains of bacteria in order to determine their antimicrobial activity. All the bacteria tested were inhibited by G.O and a much wider range (550-fold) of MIC values from 0.01-5.5mg/ml (Table 3.1a, b) was obtained, than for G.P. The most sensitive organism identified from Table 3.1d was *L. monocytogenes* (2 strains, mean MIC of 0.02mg/ml) and the least sensitive organism was *S. enteritidis* (3 strains, mean MIC of 3.78mg/ml).

These results contradict the findings of Cavallito *et al.* (1944) and Petricic *et al.* (1978), who stated that it is the thiosulphinates (allicin) of fresh garlic (and garlic powder preparations), which show significant antimicrobial activity and not the garlic sulphides of G.O. The overall MIC range for all the bacteria screened is shown in Table 3.1d. MIC values regularly showed higher values at 48 hours than at 24 hours incubation (37 cases out of 69 organisms tested). This could indicate a decline in microbial sensitivity with respect to time, although experiments to test this possibility do not support this (Section 3.3.4). It is therefore likely that this decline resulted from a progressive loss of G.O and hence antimicrobial activity from the medium (See p215).

It was observed that considerable variation in MIC values occurs within strain isolates of a species, although unlike the situation with G.P, the MIC ranges are far less than the G.O range of all bacteria. Examples are *E. coli* (18 strains), MIC range of 0.34-5.5mg/ml (x16); *L. acidophilus* (5 strains), MIC range of 0.17-2.75mg/ml (x16); *Staph. aureus* (5 strains), MIC range of 0.34-2.75mg/ml (x8); *B. subtilus* (4 strains), MIC range of 0.01-0.17mg/ml (x17); *S. enteritidis* (3 strains), MIC range of 0.34-5.5mg/ml (x16). The wider range of sensitivity of all species tested as compared to G.P, and the relative species-specificity of the sensitivity observed irrespective of strain, suggests that G.O has a more selective antimicrobial action

than G.P (as shown in Tables 3.1d, e). This may have important practical utility in that certain pathogenic gut bacteria, such as *L. monocytogenes* and *Y. enterocolytica* (Table 3.1a, b) proved to be very sensitive to G.O, whereas bacteria which are typically commensal or beneficial to the digestive system, such as *E. coli*, *E. aerogenes*, *Staph. aureus* and *L. acidophilus* (Tables 3.1a, b), are less sensitive.

The wider range of sensitivities recorded may be a consequence of G.O being comprised of a number of different potentially antimicrobial sulphides whereas the antimicrobial action of G.P has been almost entirely attributed to the single component, allicin (Hughes & Lawson, 1991). Other potential explanations stem from the lower solubility of G.O than G.P in aqueous systems such as TSB, which leads to a G.O diffusion gradient within the MIC tubes (See p214). For example it is possible that bacteria which are motile may remain higher up the MIC determination tubes away from the concentrated portion of the G.O, and hence grow and proliferate, which would not occur if the bacteria remained at the bottom of the tubes.

As for G.P, the MIC results for Gram +ve and -ve bacteria (Table 3.1d) indicated that as groups they display similar sensitivities to G.O. 5 Gram +ve species; *Bacillus spp*, *L. acidophilus*, *L. monocytogenes*, *Staph. aureus* and *Streptococcus spp*, showed an MIC range of 0.01-2.75mg/ml, whilst 8 Gram -ve species, all showed an MIC range of 0.01-5.5mg/ml.

G.O viability experiments were also performed with a variety of organisms and similar results to those with G.P observed. It was shown that, as compared to the control, concentrations below the "MIC" value result in an increase in the static population or "lag" phase before the cell population reproduces. In the presence of higher G.O concentrations, following the lag period there is a death phase followed by a levelling off period, the timing of which also appears to be concentration-

dependent. These results showed that G.O has microbiocidal effects as well as population growth inhibiting effects, indicated by the MIC results. The higher the concentration of G.O the greater the proportion of the cell population that is killed and the longer the period of time within which population decline or stabilisation persists (*E. aerogenes* (3), Figure 3.2a).

It was noted that the concentration of G.O required to inhibit growth of *E. aerogenes* from the MIC method (0.68mg/ml) and that from time-dependent cell viability studies (between 0.68 and 1.37mg/ml) regularly differed, such that the MIC concentrations were always lower than the viability concentrations. These differences were also observed for a number of other bacteria screened and possibly result from differences in experimental technique. In particular the repeated disturbance of the medium for sampling purposes in the viability studies may have increased G.O loss and therefore increased the initial G.O concentration required to inhibit growth. Alternatively the disturbance may have enhanced contact between G.O and bacteria, with reverse effect. In practice the first possibility seems the most likely (based on results obtained in Sections 3.6.5 and 3.7), as the G.O concentration required to produce inhibition of population growth was higher in the case of the viability studies.

It was also noted from the viability studies (Figures 3.2a, b) that the *E. aerogenes* cell population eventually exhibited growth after a period of time exposed to G.O at a 1.37mg/ml concentration (two-fold greater than the MIC value). Similar observations were made in the viability studies with individual "pure" sulphides (Figures 3.4.1.2a, b). As in the case of MIC results, these results are very probably attributable to the progressive loss of G.O vapour from the microbial growth medium.

It was shown that G.O exhibited antimicrobial activity in all of the various chemical environments assessed (Section 3.3.3) and that the activity was similar except in extreme non-physiological conditions, such as 0.5M sodium bicarbonate, where the activity of the G.O was masked by the biocidal effect of sodium bicarbonate on the cells. It was noted that the G.O-effected cell death rate in each of the media varied according to the particular medium, such that in non-growth environments such as saline and SDW slightly slower death rates were observed.

4.3 Antimicrobial Effectiveness Of G.O In Synthetic Intestinal Model Systems

In order to evaluate the effects of G.O within the intestine, initial work was performed with two types of synthetic intestinal fluids (simple, SIF and complex, CIF) which model the basic composition of the intestinal fluid (Section 3.5.1).

Initial pure culture experiments in SIF, CIF and TSB with *E. coli* (40) in the absence of G.O (Figure 3.5.1b), indicated that of the two simulated environments SIF exhibited a negative effect on the growth and viability of the bacterium (in terms of an initial decline in viability and a slower growth rate) as compared to TSB (or CIF) suggesting that in SIF the bacterial cells appear to be under stress and could therefore be more susceptible to the antimicrobial activity of G.O. Similar inhibition in comparison with TSB was also observed in CIF when *Shig. sonnei* and *L. monocytogenes* (Figure 3.5.1.1a) species were investigated. However in this medium such effects were deemed more acceptable since they were probably a consequence of the components of CIF (such as bile salts), which are likely to be found *in vivo*.

The antimicrobial activity of G.O was tested by triplicate MIC and pure culture viability experiments in SIF and CIF and compared to that in TSB (Section 3.5.1). Results indicated that differences in antimicrobial activity of G.O were expressed in both SIF and CIF when compared to TSB. On average lower MIC values were

obtained in both media for the same bacteria (Table 3.5.1a). In addition, from the viability studies, it was shown that in SIF (Figure 3.5.1e) the response to G.O of the bacteria tested was uncharacteristic of the trends observed in all the other environments (TSB, MSM, Section 3.3.3). In view of this and taking into account the simple composition (phosphate buffer, pancreatin, sodium hydroxide and distilled water) of SIF it was considered that the value of using SIF as a model environment for the intestinal tract was inferior to that attained by the CIF.

Pure culture viability studies in CIF were performed in the presence of various G.O concentrations (Section 3.5.1.1, Figures 3.5.1c, e, 3.5.1.1b-g) and in the case of *E. coli* (40) and *S. typhimurium* (434) were found to be comparable to those of TSB. Differences in the pattern of G.O activity were observed, with *L. monocytogenes* and *Shig. sonnei*, where a lower concentration of G.O was required to kill or inhibit growth. These differences may be due to the presence of growth inhibitory factors in CIF such as bile. It has been shown that bile, or its component (conjugated/ deconjugated) acids exhibit antibacterial activity depending on the concentration present and the susceptibility of the given organisms. Floch *et al.* (1971, 1972) showed that deconjugated bile acids are more inhibitory to bacteria than the conjugated forms, inhibiting Gram +ve bacteria but having little effect against Gram -ve bacteria. In addition, incorporation of G.O sulphides into bile salt micelles may allow the sulphides to become water soluble (Coleman, 1987), thereby enhancing the antimicrobial property of G.O, against certain bacteria, by increasing the availability of G.O to cells.

One of the main objectives of this project was to determine whether the presence of G.O in the intestinal tract can exert its effect against incoming foodborne pathogens whilst allowing the natural bacteria present in the intestine to continue to thrive. Initial simple mixed culture experiments with *E. coli* (40) (an example of a natural inhabitant of the intestine) and *L. monocytogenes* (433) (an example of a

food-borne infectious bacterium) were performed in TSB and CIF in order to determine G.O effectiveness on a mixed culture of bacteria (Section 3.5.1.2, Figures 3.5.1.2a, b). In TSB, it was observed that in the absence of G.O both organisms grow and proliferate within the mixed culture environment and the initial cell population growth rates of both organisms were similar to those obtained in pure culture. However a decrease in the final population size of both organisms was observed when compared with the pure culture studies and this could be explained in terms of competition between both organisms for available nutrients. On the basis of the sensitivities to G.O of both organisms observed in pure culture it was hypothesised that within a mixed culture the presence of G.O would have a greater effect on the *L. monocytogenes* cell population than on the *E. coli* cell population at the concentrations chosen. This effect was observed though differences were observed between the effect on individual populations in the mixed culture to those in pure culture. Thus, at the highest G.O concentration used (0.68mg/ml), both *E. coli* and *L. monocytogenes* initially decrease in cell population size, which was not observed in pure culture. Also the *E. coli* cells were able to overcome this initial decline and grow to a final population size greater than that of the inoculum level. In contrast, the *L. monocytogenes* cells showed a more significant population decrease (than *E. coli*) but not to the same extent as in pure culture where complete kill was observed for the same G.O concentration after 24 hours. Thus, assuming no variation in sensitivity from experiment to experiment, within the mixed culture the *E. coli* cells appear to protect *L. monocytogenes* cells from the full antimicrobial activity of the G.O. This effect may be due to absorption of G.O by *E. coli* cells and thus effectively reducing the available G.O concentration.

In CIF mixed culture experiments it was observed that in the absence of G.O, *E. coli* proliferates, whereas *L. monocytogenes* does not proliferate but remains in a viable state, these observations were akin to those for the two organisms in pure culture. The lack of growth of the *L. monocytogenes* population is probably due to

the effect of the medium composition in terms of the presence of bile salts (See p228). On the basis of the individual pure culture results it was expected that G.O at the concentrations chosen would selectively reduce the *L. monocytogenes* cell population in relation to the *E. coli* cell population level. In the presence of the highest G.O concentration (0.68mg/ml) similarities to the pure culture results were again observed in the mixed culture studies. Thus, the growth of both bacteria is inhibited and cell death occurs. In the case of *E. coli* cells complete kill is not achieved, however the *Listeria* cells are steadily killed by the presence of the G.O over the 24 hours. Comparison of the initial cell death/growth rates of the two organisms in pure and mixed cultures within CIF indicated similarities for *E. coli*, however the initial cell death rate of *L. monocytogenes* appeared significantly different from that in pure culture.

Similarities in results were obtained in the CIF model to those of TSB, though both organisms appear to be more sensitive to the antimicrobial effects of G.O in CIF than in TSB (also observed in pure culture studies). However at 0.68mg/ml G.O, differences between the two media were observed. In CIF complete kill of *L. monocytogenes* was achieved accompanied by a significant decrease in the *E. coli* cell population size, whereas in TSB it appears that the subsequent growth and proliferation of *E. coli* cells (after the initial decrease) may protect the *L. monocytogenes* cells at this G.O concentration.

Investigation of the effect of G.O in simple mixed cultures was continued with *L. acidophilus* (NCFM Gilliland) (an example of a natural inhabitant of the intestine) and *L. monocytogenes* (433), in MRS broth (Section 3.5.2). In pure culture both organisms readily grew in the MRS broth. As a mixed culture in the absence of G.O it was observed that the *L. acidophilus* cells grow and proliferate, whereas the viable cell population size of *L. monocytogenes* initially increases during the first 4 hours but then subsequently declines to the extent of complete kill 24 hours after

inoculation. These results were similar to the findings of Rees *et al.* (1993), who worked with a mixed culture of *E. coli* and *L. acidophilus.* They showed that *L. acidophilus*, which produced lactic acid during the growth phase, lowered the culture pH to 5.0 after 6 hours, so explaining the initial inhibition and subsequent death of the *E. coli* population. A similar reduction in pH was observed for the *L. acidophilus* and *L. monocytogenes* mixed culture presented in this thesis. It is known, however, that *L. monocytogenes* viability is not affected by the low pH values in the region of pH 3.5 to pH 7.0 generated as a consequence of homolactic fermentations (Hill, unpublished observations). Consequently the loss of viability of the *L. monocytogenes* population in the mixed culture must be attributed to another factor other than the low pH.

Lactic acid bacteria produce a variety of metabolic products known as bacteriocins, which are capable of interfering with the growth of other microbes. These have been defined as proteins having a narrow activity spectrum and a bactericidal mode of action against Gram +ve and -ve bacteria including known food pathogens (Vanderbergh 1993). It is thus possible that the observed loss of *L. monocytogenes* cell population viability in the presence of *L. acidophilus* may be due to the production of such a bacteriocin. Indeed, Barefoot & Klaenhammer (1983) and Harris *et al.* (1989) identified lactacin B, a bacteriocin produced by *L. acidophilus* to be inhibitory to *L. monocytogenes.* Alternatively lactic acid bacteria may produce other non-bacteriocin inhibitory compounds such as diacetyl, acetaldehyde, organic acids and hydrogen peroxide all of which interfere with the growth of *Listeria spp* (Schlyter *et al*, 1993).

In the presence of G.O, differences in the response of the two organisms in mixed culture to that in pure culture were observed. The *L. acidophilus* cells grow and proliferate (at slightly faster initial growth rates than in pure culture) at all G.O concentrations including the highest concentration of 0.34mg/ml. In contrast for

the *L. monocytogenes* cells, inhibition of growth was observed at the lowest G.O concentration of 0.04mg/ml while at concentrations greater than 0.08mg/ml cell death commenced immediately after inoculation and for all G.O concentrations (as in the absence of G.O), complete kill of the *L. monocytogenes* was observed after 24 hours. These results complement the findings of Rees *et al.* (1993), for the effect of G.P on *L. acidophilus* and *E. coli* mixed cultures. It can be concluded that in MRS G.O can selectively enhance the reduction of the number of live and potentially infectious *L. monocytogenes* cells, yet still allow the growth of *L. acidophilus.*

These studies provided further insight into the antimicrobial properties of G.O. Thus, in a variety of media including biological growth (TSB, MSM and MRS) and non-growth (SDW, Phosphate buffer) media and as discussed here, also in the simulated intestinal fluids (SIF and CIF) G.O is seen to be an effective antimicrobial. Only fairly minor variations in effectiveness are apparent for the same organisms in different environments (See p228).

Although it is possible that these results differ from those which occur in the intestinal tract itself, as in many varied media G.O gives an essentially similar effect, it is reasonable to infer that a similar effect would be obtained there as well. However it is extremely difficult to model the extreme complexity of the intestinal environment. In particular this complexity consists of; A) the large and highly varied organic content (especially proteinaceous material) of the intestinal fluid (which includes both digesta and secreta) which is not represented in the simple model systems AND B) the large and diverse microflora. It was, therefore, decided to assess the antimicrobial nature of G.O in the most realistic environment available, actual samples of human intestinal contents.

4.4 Antimicrobial Effectiveness Of G.O In Intestinal Fluid

A realistic laboratory-based simulated "real" gut environment was achieved by the use of ileostomy effluent (Methods Section 2.1.4.3).

The physiological nature of the ileostomy samples is discussed in the results chapter (Section 3.5.3). Microbiological analyses of the numbers and types of bacteria present in a variety of ileostomy samples were performed. It was found that there was a relatively lower viable microbial count of 2-4x10^6 cells/ml contents in the 1-7 day post-operation samples compared to those of the more established 1-10 year post-operation samples (6x10^8 cells/ml contents). This difference was thought to be due to either; 1) a disturbance in the microbial flora as a consequence of operation. The bacterial flora of the intestine is controlled by the anatomy and physiology of the intestinal tract (Drasar, 1974) and therefore any alteration such as surgery will alter the physiology and hence alter the initial microbial composition OR 2) the influence of drug treatment after operation. All the 1-7 day post-operation patients were undergoing drug therapy for; duodenal, gastric and stomal ulcers by treatment with cimetidine, ranitidine, carbenoxolone sodium and for acute/ chronic diarrhoeas by treatment with codeine phosphate, sulphasalazine and prednisolone. It is known that a number of these drugs have direct effects on the intestinal secretions (Hardey, Personal Communication) especially secretions of the gall bladder, that is bile acids which may even increase gastric acidity.

The viable count results for ileostomy fluid from 1-10 year post-operation patients (Table 3.5.3a) are in accordance with those published by Gorbach *et al.* (1967c) and Finegold *et al.* (1970) of 10^7cells/ml and 10^8cells/ml respectively. Both authors indicated that the numbers and types of bacteria present in ileostomy effluents was significantly greater (upto 80 times) than that of the normal terminal ileum, suggesting that the production of an ileostomy stoma appears to alter the microflora and allow certain microorganisms to increase in population size, with the end result of the

which may have entered the fluid from the skin surface. The presence of oxygen at any point from the discharging of the IF to the enumeration procedure for microorganisms may explain the lack of detection of strict anaerobic organisms. Although disadvantages have been identified in using ileostomy fluids, it is believed that this was a worthwhile model for developing a "near" *in vivo* situation for monitoring the antimicrobial effectiveness of G.O.

In order to determine the antimicrobial activity of G.O within IF, viability studies with *L. monocytogenes* (433) were performed (Section 3.5.3, Figures 3.5.3b-e). Incubation of the IF, in the absence of G.O, allowed the natural intestinal bacterial population to grow and proliferate from 10^8 to $10^{9.5}$ cells/ml over a period of 24 hours. *L. monocytogenes* when inoculated into the IF in the absence of G.O exhibited a slow decline in viable cell population size with respect to time but was still present in significant numbers ($10^{4.5}$ cells/ml) after 24 hours. It was hypothesised that this decline in the *L. monocytogenes* cell population size in the absence of G.O was due to either; 1) the antagonistic effect of the natural microflora present, for example by bacteriocin production OR 2) the effect of non cellular components of the ileostomy fluid such as the presence of proteins (enzymes) and bile. The latter has certainly been shown in this study to inhibit the growth of *L. monocytogenes* at a concentration of 5.6g/L (Figure 3.5.1k). The concentration of bile in the jejunum/ ileum is variable but is reported to be on average 10mmol/L (Gorbach & Tabaqchali, 1969) and so higher concentrations of bile in conjunction with other components, such as mucus, lysozyme, bacterial by-products and fatty acids (Dixon, 1960; Drasar *et al.* 1969) may account for the observed loss of *L. monocytogenes* viability.

In order to test the effect of the resident microflora present in the ileostomy effluent upon the viability of *L. monocytogenes* (433), it was hypothesised that removal of these natural organisms may result in growth and proliferation of *L. monocytogenes* cells (Figures 3.5.3f, g). After autoclaving the IF it was observed that the inoculated

L. monocytogenes cell population grows and proliferates, suggesting that the anti-*Listeria* components of fresh IF were due to either the presence of the natural viable bacteria or to heat sensitive medium components. After centrifuging the IF (causing a reduction in the natural microflora of approximately 100-fold, without causing changes to the natural physiology in terms of chemical changes influenced by autoclaving), the inoculated *L. monocytogenes* cell population initially grew and proliferated. However the final population size declined over the 24 hour period in conjunction with an increase in the resident flora population. It would appear from these results that the presence of the normal microflora caused the loss of *L. monocytogenes* viability in fresh whole ileostomy fluid.

The presence of G.O in fresh IF (Section 3.5.3, Figures 3.5.3b, e) at a concentration of 0.17mg/ml, allowed the growth and proliferation of the natural microflora (although at a slower growth rate compared to the absence of G.O) to a final population size of $10^{8.2}$ cells/ml. In contrast, the number of viable *L. monocytogenes* cells decreased at a much faster rate than those in the absence of G.O, such that at 1440 minutes following the addition of G.O, the viable *L. monocytogenes* cells were only detectable at a level of $10^{3.1}$ cells/ml. It should be noted that the effectiveness of G.O at low concentrations was masked by a greater antimicrobial effect of the natural flora within the IF (comparison of Figures 3.5.1j and 3.5.3e). The effects of G.O were most obvious during the first 4 hours which was prior to the antimicrobial effect of the natural flora and during this period only the effect of high (0.34mg/ml) G.O concentrations on the *L. monocytogenes* cells are observed.

Comparison of the effectiveness of G.O within IF, TSB and CIF was made in terms of the effect of G.O on the initial cell death rate of *L. monocytogenes* (Section 3.5.3, Figure 3.5.3h). The results indicated that the cellular response of *L. monocytogenes* in IF was similar to that of CIF, in that increases in G.O concentration produce comparable increases in death rates for TSB, CIF and IF, but inhibitory effects of

G.O in CIF and IF were not observed, as in TSB, due to the greater inhibitory effects of those media. This observation indicated that CIF was an appropriate synthetic model system to use prior to the use of IF. However the effectiveness of the natural flora on the *L. monocytogenes* cells in IF led to a well defined initial cell death rate compared with the other two media. The results obtained indicate that the antimicrobial properties of G.O, in terms of selectively reducing or eliminating *L. monocytogenes* cells from the IF will allow the natural intestinal bacteria to proliferate. The presence of G.O appears to enhance the rate of loss of *L. monocytogenes* viability over that effected by the natural microflora and thus reduces the residence time of a potentially infective dose of *L. monocytogenes* in IF.

4.5 Activity Of Garlic Products With Respect To Antimicrobial Mechanisms

It has been shown in this study that both garlic products, G.P and G.O are antimicrobially effective against all the bacteria screened within a variety of environments. These findings contradict those of Cavallito *et al.* (1944), who stated that it is the thiosulphinates of garlic and not the sulphides (which can be formed from them) that are antimicrobially active. This belief has restricted research into the antimicrobial activity of garlic oil and its component sulphides. The actual mechanism of action of fresh and freeze-dried garlic extracts still remains largely unknown. However a number of studies have been performed in attempts to elucidate the antimicrobial modes of action of these thiosulphinate-containing extracts (See Introduction).

Extracts of fresh and freeze-dried garlic preparations (such as G.P) are complex mixtures containing methylallyl, allylmethyl, dimethyl and diallyl thiosulphinate (allicin) and small amounts sulphides containing 1-6 sulphur atoms (Hughes & Lawson, 1991). The main active antimicrobial component, allicin, represents only a small proportion of the total weight of G.P used in the MIC determinations (Lawson *et al.*, 1991a). Spectrophotometric results for the determination of allicin

obtained for G.P in this thesis (Section 3.6.1) indicated an allicin concentration of 11mg/g dry weight G.P. Using this value and MIC data for G.P obtained in this study the equivalent MIC values for allicin would be in the range of 34.3-137.5µg/ml. These values are comparable to the MIC values obtained by Rees *et al.* (1993) of 10-480µg/ml for a garlic powder preparation and Hughes & Lawson (1991a) of 75-150µg/ml for garlic clove homogenates against *E. coli* and *Staph. aureus.* However estimates from freeze-dried or garlic clove preparations are higher than those values exhibited by pure allicin, as shown by Delaha *et al.* (1985) who observed MIC values of allicin in the range of 1.34-3.35µg/ml when tested against a variety of *Mycobacterium* species and Chowdhury *et al.* (1991) who indicated that 0.4µg/ml allicin inhibited the growth of *Shig. flexneri.*

Although it is not clear how allicin or the other thiosulphinates of garlic exert their effects upon microbial cells, a number of proposals have been suggested. Allicin may react with sulphydryl groups (Cavallito *et al.*, 1944; Small *et al.*, 1947; Wills, 1956). The majority of sulphydryl and disulphide groups in cells are proteins, unless sequestered in the matrix of its protein the sulphydryl group is the most variably active group found in cells. Most enzymes contain sulphydryl groups which may be involved in direct catalysis, maintenance of structural integrity of the enzymes, binding of substrates and also in allosteric binding, thereby affecting regulation of the enzyme (Jocelyn, 1972).

Small *et al.* (1947), by synthesis of a series of alkylthiosulphinates with the general formula R-SO-S-R, demonstrated that the -SO-S- group was essential for bactericidal action of thiosulphinates. Wills (1956) showed that the -SO-S- grouping of allicin was essential for the inhibition of the activity of a number of known sulphydryl enzymes including succinic-oxidase and urease enzyme activities. Wills (1956), observed that concentration ranges of $5x10^{-4}$-$5x10^{-5}$ M allicin completely inhibits many important metabolic enzymes, especially those having reactive SH-groups. Comparable evidence has been obtained in this study (Section

3.8). Thus a 25mg/ml concentration of G.P, containing approximately $2x10^{-4}$ M allicin, inhibited the enzymatic activities of alcohol and lactate dehydrogenase (ADH and LDH), although it should be noted that total inhibition of LDH was not achieved. Further evidence to support the implication of SH-groups in garlic thiosulphinate action was observed by Rao *et al.* (1946) and Barone & Tansey (1977), who showed that cysteine inhibited the antimicrobial effectiveness of allicin. Studies involving the interaction of garlic and thiol-containing compounds suggested that thiosulphinates, such as allicin, present in aqueous garlic extract exert their effect on the oxidation of thiol groups present in the essential proteins of the microorganism. Cavallito (1946) suggested that the active components may react with essential sulphydryl groups of bacterial enzymes with consequential loss of activity or may react with the SH-groups in cysteinyl residues, as these are joined at the end of a growing polypeptide chain during protein anabolism, such that allicin might block further growth of the protein along the chain by producing cysteine "dead ends". The lack of changes in sensitivity of bacteria to G.P experienced in Section 3.1, in that there was no clear difference between Gram +ve or -ve bacterial responses with respect to allicin is not a view supported by the findings of Tynecka & Gos (1973) and Small *et al.* (1947).

Schaechter & Santomassino (1962) observed that *Escherichia coli* in log phase cultures will lyse in the presence of extremely small amounts of some SH-combining compounds, thereby losing intracellular components. Ghannoum (1988; 1990), observed that aqueous garlic extracts, affected the structure and integrity of the outer envelope of *Candida* cells causing extensive leakage of the cytoplasmic contents from the cells correlating to morphological and ultra-structural changes in the yeast. This may arise as a consequence of the action of garlic against sulphydryl enzymes involved in lipid biosynthesis, a hypothesis supported by Focke *et al.* (1990), working on acetyl-CoA synthetase.

It can therefore be tentatively concluded that the antimicrobial mode of action of allicin in terms of its biocidal and bacteriostatic activity may be to disrupt cell metabolism by; 1) inactivating proteins by oxidation of essential thiols to disulphide; 2) competitively inhibiting the activity of non protein sulphydryl compounds such as cysteine (required for protein synthesis) and glutathione (required as a cofactor for a number of enzymes) by combining with them AND 3) non-competitively inhibiting enzyme function by oxidation of the binding to SH-groups at allosteric sites. Thus reaction with SH-groups important for microbial metabolism and consequential microbial growth and proliferation may be the mechanism by which allicin-containing aqueous extracts of garlic (including G.P) inhibit the growth of, and kill, microbial cells.

The extensive findings of this thesis that G.O exhibits antimicrobial activity led to investigations of which, if any, of the sulphides present were responsible for the antimicrobial activity in G.O. MIC determinations of four commercially available G.O sulphides (DMD, DMT, DAS and DADS), indicated that each sulphide exhibited varying degrees of antimicrobial activity (Table 3.4.1.1a), suggesting that the individual sulphides present within G.O are at least partly responsible for the expressed antimicrobial activity of G.O. A possible explanation already suggested is that volatilisation of the individual sulphides may take place at different rates. Both MIC and viable count data for the commercial sulphides DADS, DAS, DMD and DMT suggests that none of these fully account for the observed antimicrobial activity of G.O. However if the differences in antimicrobial activity are taken at face value other sulphides, not tested but known to be present in G.O and/or the unidentified non-sulphide component(s) may be its major active antimicrobial component. Alternatively, synergistic effects between sulphide components and/or other unidentified components may account for the higher antimicrobial activity of G.O as determined from MIC studies. Non-sulphide unidentified materials account for approximately 20-30% of the total G.O

composition.

Garlic sulphides have different molecular sizes and boiling points (Section 3.6.5). It is therefore likely that antimicrobial activity associated with the "pure" sulphides tested was lost at different rates from one another and from G.O. Thus experiments which evidenced volatilisation indicated differences in rate of loss of antimicrobial activity (Section 3.7). This explanation also fits the observation that the smaller (more volatile) "pure" sulphides (DMD and DAS) tested by MIC determinations (Section 3.4.1.1) showed lower apparent antimicrobial activity than G.O, whereas the relatively large (less volatile) "pure" sulphides displayed a higher apparent antimicrobial activity.

Studies of the effect of the individual sulphides DADS (0.625-5mg/ml) and DAS (2.5-5mg/ml) on *E. aerogenes* cell population viability with respect to time (Figures 3.4.1.2a, b) indicated concentration-dependent bacteriostatic and bacteriocidal effects, similar to those observed with G.O. Thus following an initial decline in the population size, increases in the bacterial cell population were observed. Discussion of the possible explanations of this phenomenon are given in Section 3.2 (p88). A study comparing antimicrobial activity of individual sulphides to that shown by G.O was also performed in terms of the effect on initial cell death/growth rates. A 4.4mg/ml DAS concentration produced an *E. aerogenes* death rate in TSB of -0.204 $mins^{-1}$ (Figure 3.4.1.2c). The same death rate required a concentration greater than 25mg/ml of G.O (Figure 3.2f) containing only around 0.39mg/ml DAS (as calculated from sulphide composition of G.O derived from GC analysis, Section 3.6.2, p176). Thus the DAS in 25mg/ml G.O is 10% of the "pure" DAS concentration which gives the same death rate as the concentration of G.O. This suggests that about 90% of the death rate due to G.O cannot be explained by any separate action of DAS. Thus DAS acting separately is only a minor antimicrobial component of G.O and consequently other garlic sulphides and/or other unidentified components

of G.O must play the major role in the antimicrobial activity of the G.O. An intriguing aspect of this comparison is that it also suggests DAS to be around five times more antimicrobially potent than G.O. This runs counter to the evidence for differences in apparent potency obtained in the (longer-running) MIC and viability experiments. It seems reasonable to explain this discrepency in terms of the relative absence of volatilisation as a major factor in the short-term (maximum of two hours) initial death rate measurements. Thus it seems that DAS may well be considerably more active antimicrobially than G.O in itself. This tentative interpretation is consistent with the apparently lower antimicrobial activity of DAS suggested by the longer term MIC and viability studies. Thus in these greater loss by volatilisation of DAS than of G.O was observed.

HPLC analyses (Section 3.6.5) indicated that these commercial preparations were not "pure". However, as each of the individual commercial sulphide preparations contained a mixture of allyl and methyl sulphides found to be present in G.O evidence that the antimicrobial nature of G.O is at least mainly due to its sulphides was obtained.

It was hypothesised that the inorganic ion concentrations of the fluid environment may affect the antimicrobial mechanism of G.O (Section 3.3.3). It has been shown that the action of certain antimicrobial agents can be influence by the osmotic effects involving the cytoplasmic membrane (Russell, 1974). Different anions and cations exert independent effects on the permeability of cell membranes, such that the activity of antimicrobials, may be increased by the presence of various inorganic salts in suitable concentrations (Bean & Das, 1966; Briozzo *et al.* 1989). However not all microbes will be affected by the same particular hypertonic solution (Csonka, 1989), that is, those organisms with a comparatively low concentration of intracellular solutes (Gram -ve bacteria) are more susceptible to plasmolysis than organisms (Gram +ve bacteria and yeasts) containing a higher

concentration of solutes (Cota-Robles, 1963). The results obtained in this thesis indicated that the existence of G.O biocidal activity is independent of whether bacterial cells are growing or non-growing as effected by the presence or absence of a carbon source (Section 3.3.3). As a reaction between inorganic salts and garlic sulphides seems unlikely, the apparent change in sensitivity of the cells to G.O in non-growth inorganic salts medium probably results from an effect of the salts upon the bacterial cells influencing the uptake or action of G.O components upon the cells.

As previous workers have failed to recognise the potent antimicrobial activity of G.O, little literature on its antimicrobial mode of action exists. Results obtained in this study for the antimicrobial activity of G.O in tryptone soy broth (TSB) indicate that tryptone may have an antagonistic effect upon the antimicrobial effectiveness of G.O, perhaps due to its content of sulphydryl-containing amino acids. Thus in MSM+glucose slightly higher death rates were observed than for the same concentrations of G.O in TSB. Since differences in G.O activity (in terms of *E. aerogenes* cell population death rates) were shown between the two growth media, TSB and MSM, the effect of tryptone additions to MSM were studied in the presence of lethal concentrations of G.O (Figure 3.3.3c). It was shown that in the absence of G.O, all concentrations of tryptone (5-15g/L) stimulated *E. aerogenes* growth, while in the presence of G.O, growth of *E. aerogenes* cells was directly related to tryptone concentration and the lowest tryptone concentration (5g/L) resulted in death over the 24 hour period of experimentation. Thus the greater the tryptone concentration, the greater the reduction in the bacteriocidal properties of G.O. The possibility that the antagonistic effect of tryptone against the antimicrobial effectiveness of G.O was due to the presence of sulphydryl containing amino acids was considered. Incorporation of L-cysteine into MSM+glucose medium in the presence of G.O (Figure 3.3.3d) revealed that cysteine alone has an antagonistic effect on the antimicrobial effectiveness of G.O, such that at cysteine concentrations

of 25mM, the biocidal activity of G.O was no longer observed and cell population growth occurred.

Both of these results are comparable to those of Rao *et al.* (1946), for the effect of cysteine on the antimicrobial effectiveness of allicin. Thus inhibition of the SH-groups of key molecules such as enzymes dependent for their cellular function upon their SH-groups may be the mechanism by which both aqueous garlic extracts and G.O inhibit the growth of, and kill, microbial cells. Results presented in this thesis therefore may suggest a comparable target site for the mode of action of sulphides present in G.O to that evidenced for thiosulphinate (allicin) containing garlic preparations (such as G.P). This possibility is further supported by results for the effect of G.O and two individual sulphides on the known sulphydryl enzymes, ADH and LDH (Section 3.8), which indicated that G.O, DADS and DMD all act in a similar manner against both enzymes to those effects observed with allicin-containing preparations (Wills, 1956). As mentioned previously (p240), allicin may interact with functional sulphydryl groups of the enzymes by oxidation of the binding to SH-groups at allosteric sites, or with the sulphydryl groups in cysteinyl residues by forming a disulphide bridge either between two protein molecules, two sulphydryls of the same molecule or a mixed type. The sulphydryl groups of both ADH and LDH are near or at the active site of the enzyme (Jocelyn, 1972), which would explain the observations of inhibition of activity of both enzymes upon addition of G.O and the two sulphides. It should be noted that the concentration of G.O required to inhibit enzyme activity is lower (0.0013mg/ml for ADH and 0.0027mg/ml for LDH) than that required to inhibit cellular growth (0.34-2.75mg/ml). This may suggest that the cellular target sites for antimicrobial action are less sensitive to G.O than are the enzymes studied in this thesis or that the bacterial cell wall provides restricted access of hydrophobic sulphides into the cell.

Further evidence to support the implication of SH residues as target sites for G.O sulphides comes from experiments incorporating glutathione (a sulphydryl

group blocking agent) into enzyme/sulphide reaction mixtures. It was shown that pre-incubation of the ADH with glutathione appears to protect the enzyme against the effects of G.O and the longer the period of incubation of enzyme and GSH, the more active the enzyme is or the less inhibitory the effect of G.O becomes. However it should be noted that incubation did not entirely prevent enzyme inhibition, as was noted by Ghannoum (1988) working on the anticandidal action of garlic and by Kumar *et al.* (1991) working on the inhibition by diallyl di- and trisulphides of 3-hydroxy-3methylglutaryl CoA (HMG CoA) reductase. The latter workers showed that DADS irreversibly inactivated HMG CoA reductase, due to a rearrangement of thiol-disulphide groups.

George *et al.* (1973) indicated that protein synthesis in larvae was significantly inhibited by pretreating mosquito larvae with garlic oil and diallyl disulphide. George & Eapen (1974) have provided some evidence for the mode of action of G.O, showing G.O and DADS to be similar in action to dinitrophenol (DNP) as effective inhibitors of oxidative phosphorylation *in vitro* in hepatic mitochondria of mice. They showed that phosphorylation was inhibited to a greater extent than respiration but was dependent upon the substrate utilised (such that, respiration was more susceptible to the action of G.O during the oxidation of glutamate), suggesting that G.O acts primarily as an uncoupler of oxidative phosphorylation.

The results presented in this thesis for G.O and the two individual sulphides (DMD and DADS) when compared to those for G.P, provide some evidence for a similar mode of action of G.O to that of allicin. Thus antimicrobial activity can be explained by an impairment of enzyme systems including those involved in production of cellular energy and synthesis of structural components. The different sensitivities expressed by bacteria to G.P and G.O may stem from; 1) the types of active antimicrobial components within the two products, that is the thiosulphinates (mainly allicin) in G.P compared with the variety of sulphides present in G.O, a majority of

which have been shown to possess antimicrobial activity (Yue *et al*, 1984); 2) the physical availability of either type of antimicrobial components to cells AND 3) the concentration of the active components. However recent investigations by Lawson (1993) suggest the possibility that the similarities of action observed between G.O and G.P stem from the conversion of their different sulphur compounds to the same molecule. Thus Lawson found that incubation with blood quickly converts both allicin and sulphides (DATS, DADS) to allyl mercaptan. If such a process occurs in microbiological media/cells then the differences in antimicrobial activity between G.O and G.P may be explicable in terms of 2) and 3) above only (that is, not in terms of any differences in the initial chemical composition of G.O and G.P which is suggested in 1).

In summary evidence suggests that allicin and presumably the sulphides present in G.O probably inhibit the enzymes present in the periplasmic and cytoplasmic space of bacteria by blocking the sulphydryl groups of proteins. If these proteins are necessary requirements for the growth and proliferation of microorganisms, then the presence of allicin or the sulphides of G.O will result in the loss of cell growth and viability.

4.6 Evaluation Of The Potential Use Of Garlic Products As Antimicrobial Agents *In Vivo*

The results of the MIC (Section 3.1) and viability studies (Section 3.2) show that both G.P and G.O exhibit a broad-spectrum of antimicrobial activity. At low concentrations both products exert a bacteriostatic (population growth inhibitory) effect whilst at high concentrations they exhibit a bacteriocidal effect.

4.6.1 Comparison Of G.P And G.O With Respect To Antimicrobial Effectiveness

Comparison of the antimicrobial activity (based on MIC results, Section 3.1.1) of G.P and G.O on a "weight of product" basis indicated that G.O was plainly more potent than G.P. This was especially so against the organisms *L. monocytogenes* (a foodborne pathogen), where G.O was 312.5 times more potent than G.P, and *B. fragilis* (a natural microorganism of the GI tract), for which G.O was determined to be 156.25 times more potent than G.P. However on correlating the "weight of product" with the stated commercial products recommended daily dose (recommended mg dose/day for G.O=2, for G.P=800), a factor which reflects the relative potency/recommended dose, considerably higher values were obtained for G.P than G.O, thus on this basis it would appear that the thiosulphinates present in G.P are more potent than the sulphides present in G.O. Although it was noted that variation occurs with species of microorganism, it was shown that for *L. monocytogenes* and *B. fragilis* the recommended dose for G.P is 1.28 and 2.56 times more potent than the recommended dose of G.O respectively.

An alternative comparison of the antimicrobial activity of G.O and G.P was made in terms of the effects on initial cell death/growth rates (Section 3.2, Figures 3.2f, g). In the presence of G.O bacterial cell death occurred immediately (a response not observed with G.P) in which a greater initial proportion of the cell population was killed. Thus G.O appears to be more potent than G.P. In the presence of G.P, at equivalent concentrations, the bacteria entered a "lag" phase in which all populations remained relatively steady or grew slightly before cell death was observed. This could be because G.P initially affected the metabolic activity of the bacterial cells prior to exerting its killing effect. An alternative possibility, in view of the evidence for ready conversion of allicin to other compounds (p221), is that this "lag" phase represents the time-period during which such compounds (such as sulphides or allyl mercaptan) are formed. Whether this occurs in the presence of

cells remains as yet untested but no evidence for it was found after incubation in microbial medium alone.

4.6.2 Classification Of G.O And G.P As Therapeutic Antimicrobial Agents

Classification of materials as antimicrobial agents useful in the treatment or prevention of any given disease depends upon:-

1) antimicrobial sensitivity of the infecting organisms to the particular garlic agent;

2) an absence of side affects of the antimicrobial agent resulting from direct toxicity to mammalian cells or to adverse effects upon the symbiotic microbiota associated with human tissues;

3) any biotransformations of the antimicrobial agent that occur *in vivo*, that are relevant to whether the agent will remain in its active form in the gut for a sufficient period of time to be effective;

AND 4) chemical properties of the antimicrobial agent that determine its distribution within the body, that are relevant to whether or not adequate concentrations of the active antimicrobial chemical will be able to reach the site of infection in order to inhibit or kill the pathogenic microorganisms causing the infection (Croshaw, 1977).

The following discussion attempts to relate the findings of this thesis and those of others to each of the above factors so as to allow conclusions regarding the suitability of garlic products for the treatment or prevention of diseases to be drawn.

Cavallito *et al.* (1944), stated that it is the thiosulphinates (allicin) of fresh garlic which show significant antimicrobial activity and not the sulphides as found in G.O, this however is not a view supported by the findings obtained in this study. Thus, the antimicrobial results obtained in this study from MIC and viability studies for a range of bacteria indicated that at sufficiently high concentrations both garlic products are effective as antimicrobial agents against all the bacteria tested. MIC and

viability studies using four commercially available G.O sulphides indicated that each sulphide exhibited varying but considerable degrees of antimicrobial activity. This strongly suggests that the individual sulphides within G.O are at least partly responsible for the observed antimicrobial activity of G.O and may explain the whole of it.

In order to evaluate the sensitivity of potentially infectious microorganisms to a particular garlic product, comparison of its antimicrobial activity to known antibiotics should be performed. This is useful in order to; 1) compare and contrast the effects of the garlic preparations on intestinal flora with those exhibited by known antibiotics AND 2) to evaluate the effectiveness with respect to actual concentration which is of particular value when considering developed resistance by bacteria to antibiotics, an effect shown in this thesis not to occur with G.O against *E. aerogenes* (Section 3.3.4). Sharma *et al.* (1977) compared the antimicrobial activity of a crude garlic extract (impregnated disk containing 19.9mg) to 12 antibiotics commonly used as chemotheraputic agents (Ampicillin, 10µg, Bacitracin, 10 IU, Chloramphenicol, 30µg, Chlortetracycline 30µg, Erythromycin, 15µg, Kanamycin, 5µg, Nitrofurantion, 300µg, Oxytetracycline 30µg, Penicillin 10 IU, Polymyxin 300 IU, Streptomycin 10µg and Tetracycline 30µg) against a variety of both Gram -ve and Gram +ve bacteria. Results, in terms of zones of inhibition of growth, indicated that the extract was not as effective as the antibiotics tested, however it was shown to be potentially useful against bacteria with developed resistance to antibiotics such as *Proteus spp* and *E. coli*. Hughes & Lawson (1991) compared a pure allicin preparation to gentamycin against *E. coli* and *Staph. aureus*, determining that 28µg/ml of allicin was required to inhibit growth of both organisms compared to 0.35 and 0.19µg/ml gentamycin respectively, indicating that allicin is considerably less active against these two organisms than the antibiotic. However, Fulder (1990) stated that in comparative plate culture tests, 0.1ml fresh garlic juice (containing allicin of unknown quantity) was at least as effective as 6µg of

establishment of a new equilibrium and a distinct microbial flora.

The organisms: *Staphylococcus aureus*, *Staph. epidermidis*, *Staph. haemolyticus*, *Xanthomonas maltophilia* and *Bacillus spp* not commonly found in the intestinal tract, were identified in 3 out of 5 IF samples. These isolates probably entered the effluent as it passed through the stoma into the ileostomy bag, as such organisms are commonly associated with the skin and are able to proliferate and survive in IF after having entered the ileostomy bag. In addition it is known that *Bacteroides fragilis*, the most prevalent organism of the large intestine (90-99%), present there in large numbers (10^5-10^8/ml), is also present in the normal small intestine (Tally, 1993). However was not detected in the IF samples used in this study. Similar results for the lack of detectability of *B. fragilis* in ileostomy fluids were also found by Finegold *et al.* (1970), who suggested that when these organisms are transferred from their natural environment to artificial media, the numbers decline and eventually death occurs in the presence of small quantities of oxygen.

There are several problems encountered in using ileostomy fluid samples as representatives of the ileum in terms of the bacteria isolated. The methods of studying bowel flora used in this study, for the collection of samples and the subsequent isolation and identification of the microflora probably only account for 10-25% of the normal intestinal bacterial flora (Drasar & Hill, 1974). Improved anaerobic techniques, such as the roll-tube procedure (Moore & Holdeman, 1974), which allows for constant anaerobiosis from the time media are prepared through the entire procedure of inoculation, examination, transfer and identification would undoubtedly improve results. However, this was difficult to maintain due to the way in which ileostomy samples are collected. The samples were collected from full ileostomy bags and the length of time the sample is left in the bag (age of the sample) together with the temperature conditions may influence the nature of the samples in terms of the numbers and types of bacteria present, particularly those

penicillin, 100µg streptomycin, 50µg of chloramphenicol or 30µg tetracycline. Barone & Tansey (1977) indicated that the anticandidal component of garlic (allicin) may be effective at concentrations used by antifungal drugs, such as miconazole 2% cream, amphotericin B 3% lotion and griseofulin 1g orally. Comparative tests with G.O have as yet not been performed. Results presented in this thesis for MIC values indicated G.P and G.O in the ranges 25 down to 3.125mg/ml and 5.5 down to 0.01mg/ml respectively which are significantly higher than published data of MIC values for antibiotics (p250). It is therefore concluded from this study that G.P and G.O are not as effective as these antibiotics but this does not detract from their potential as antimicrobial agents.

Although few scientific studies have been performed in relation to the side effects of consumption of garlic, a number of commonly known side effects have been reported. The smell of garlic is often considered unpleasant (Rosin *et al.*, 1992) and after consumption, garlic breath (Minami *et al.*, 1989) and sweat odours (Doty, 1981) have been detected, the isolated components being allyl mercaptan and DADS. Sebrell (1930), reported that dogs ingesting excessive quantities of garlic and onions suffered from anaemia and jaundice and the possibility of similar effects in humans consuming excessive quantities should be studied. Caporaso *et al.* (1983) indicated that the maximal tolerable dose for volunteers was 250g of garlic (in powder extract form), producing symptoms of nausea, diaphoresis and light-headedness together with a burning sensation in the mouth, esophagus and stomach for 30 minutes after consumption. In relation to the exhibited side effects of garlic resulting from direct toxicity to mammalian cells, it should be noted that full clinical trials have not been performed, however an oral dose of 7.5g garlic powder/Kg body weight was shown by Kanezawa *et al.* (1984) to be non-genotoxic in mice. Chowdhury *et al.* (1991) however, observed that the LD_{50} of an aqueous garlic extract (containing 3.3g garlic/ml extract) was 173ml/Kg body weight and for allicin was 204ml/Kg body weight, being toxic doses to mice

and rats but showing no adverse effects on the blood serum and blood urea-nitrogen. The cytotoxic effects of garlic have been investigated by Weber *et al.* (1992), who observed that a garlic extracts (3.5 and 11mg/ml) and an allicin preparations (3.2 and 10µg/ml) were more toxic to Vero cells than to HeLa cells respectively. Rees *et al.* (1993) provided no evidence of cytotoxicity on the growth of mammalian tissue cells (Simian epithelial cell line) at garlic powder concentrations up to 10mg/ml. The toxic effects of G.O have as yet not been reported in the published literature.

The effect of garlic preparations on the symbiotic microflora of the gastrointestinal tract have been studied *in vitro* (in this study) and *in vivo* (Subrahmanyan *et al.*, 1957a. 1958 in rats; Shashikanth *et al.*, 1984 in rats). Pure culture results obtained in this study have shown that both *E. coli* and *L. acidophilus*, bacteria that naturally inhabit the intestinal tract, playing an important role in the maintenance of a healthy digestive system, appear to be less sensitive to G.O (MIC values of 1.87mg/ml and 1.95mg/ml respectively) compared with certain pathogenic bacteria such as *L. monocytogenes* (MIC=0.02mg/ml) and *Y. enterocolytica* (MIC=0.17mg/ml). *Bacteriodes fragilis*, the most prevalent organism found in the lower regions of the GI tract (90-99%) was also sensitive to G.O (MIC=0.03mg/ml), however due to the large population size (10^7-10^{12}/ml) present in the GI tract it is hypothesised that the *B. fragilis* population would be able to overcome the effects of G.O and ultimately regrow and proliferate. Hill & Maslin (unpublished data) for *E. aerogenes* indicated that the effectiveness of G.O and G.P extracts decrease at high microbial cell population levels. This view is also supported by Barone & Tansey (1977) for garlic powder extracts against *Candida albicans.*

Mixed culture experiments performed in this thesis on *L. monocytogenes* with *E. coli* or *L. acidophilus*, in a variety of synthetic media showed that G.O could selectively reduce the *L. monocytogenes* population whilst allowing the normal enteric bacteria

to survive. Similar results were obtained by Rees *et al.* (1993) studying the effect of garlic powder on a mixed culture of *L. acidophilus* and *E. coli*. In addition, results obtained using "real" gut fluid from ileostomy patients (Section 3.5.3) indicated that G.O could selectively reduce or eliminate *L. monocytogenes* cells from the IF whilst allowing the natural intestinal bacteria to proliferate. Published *in vivo* effects of garlic (and especially G.O) on intestinal bacteria are scarce. Subrahmanyan *et al.* (1957a, 1958) indicated that administration of garlic (0.3-0.4g daily) to rats along with the diet (over a 3 week period) significantly decreased the number of coliform and anaerobic bacteria from $3.5x10^4$ and $9.4x10^6$ to $2.4x10^3$ and $2.8x10^6$ respectively and increased the *Lactobacillus spp.* population size in the caecum of rats. These results would appear to suggest that in rats incorporation of garlic is likely to shift the balance of the microflora in the intestine in favour of lactic organisms, known to play important roles in the digestion and absorption of dietary materials (Mitsuoka, 1992), an effect also observed in *in vitro* experiments in this thesis (Section 3.5.2). A comparative study of raw garlic extract (0.5g/ml) and tetracycline hydrochloride (4mg/ml) on the caecal flora of rats (Shashikanth *et al.*, 1984) revealed that after a 3 day treatment period the caecal flora of both groups had been significantly reduced. However after reversion to a control diet the tetracycline fed rats reverted back to normality faster than those initially fed garlic, indicating a prolonged inhibitory action of the garlic extract on the intestinal organisms. It can thus be concluded that at least for laboratory animals, the consumption of garlic materials can have an effect on the normal intestinal flora with respect to population size and the balance between species.

The problem of determining the antimicrobial activity of a particular agent is much more complex *in vivo* than *in vitro*, it involves not only the antimicrobial agent and microorganisms but also a third factor, the host and any transformations taking place therein. The chemical environment *in vitro* is generally constant for all

members of a microbial population (except in the later stages of batch cultures). In the host however, varying environmental influences may affect the effectiveness of the garlic products against microorganisms within the intestinal tract, therefore the response of the microbial population towards an antimicrobial agent is much less uniform *in vivo* than *in vitro.*

The effect of pH on the active components of both garlic products is of importance when considering their passage through the intestinal tract, changing from an acidic pH of 1.5-3 in the stomach to a pH of 7 leading to 8 in the small and large intestine respectively (Davenport, 1982). It should be noted that no work has been published for the effect of pH on G.O components but according to Barlow (Personal Communication) the sulphides present in G.O are stable at low pH values of 2-5. Lawson & Hughes (1992) showed that no thiosulphinates are released from a garlic powder preparation at this pH and furthermore, pure allicin incubated at pH 1.2 at 37°C remains fairly stable for 5 hours (Barlow, Personal Communication). Upon entering the small intestine, the stomach contents are neutralised (to pH 6-7) and it was suggested by Lawson & Hughes (1992) that alliinase activity may be restored. However neutralisation (at pH 7-9) of garlic powder previously exposed to low pH (2-3) did not restore thiosulphinate yield. It was therefore hypothesised that low pH completely and irreversibly inhibits alliinase (enzyme) activity (required for conversion of alliin to allicin). These results indicate that a stomach acid-resistant coating on garlic powder tablets may be necessary for alliinase-mediated thiosulphinate release in the small intestine. Alliinase activity and consequent production of thiosulphinates has been shown not to be significantly affected by intestinal proteases that would normally be present in the duodenal and jejunal regions of the small intestine (Lawson & Hughes, 1992). It has however been shown (in this thesis) that the *in vitro* activity of garlic products may be impaired by the biochemical physiology of the surrounding medium and since the biochemical environment of the gastrointestinal tract is very complex, significant interference of

garlic activity would probably occur. Results obtained in this thesis using the synthetic gut fluids (SIF and CIF) compared to TSB indicated that certain bacteria (*Shig. sonnei* and *L. monocytogenes*) when in the former media appear to be more susceptible to the antimicrobial activity of G.O. This is probably due to the composition of the two fluids; SIF, containing 10g/L pancreatin and CIF, containing 5.6g/L porcine-bile extract. It is known that pancreatin contains a variety of pancreatic enzymes which may affect the bacteria present and it has been shown that bile, or conjugated/deconjugated acids present in the small intestine exhibit antibacterial activity depending upon concentration present and the susceptibility of the microorganisms (Floch *et al.*, 1971; 1972). The activities of neither garlic product in the latter section of the small intestine (ileum) have been published, however *in vitro* results obtained in this thesis (Section 3.5.3) using G.O in ileostomy fluids has indicated that G.O is effective and can selectively reduce or eliminate *L. monocytogenes* cells, allowing at least some of the natural intestinal bacteria to proliferate. The presence of G.O appeared to enhance the rate of loss of *L. monocytogenes* viability over that effected by the natural microflora and thus reduce the residence time of a potentially infective dose of *L. monocytogenes.*

In terms of biotransformations of the garlic products taking place within the intestinal tract it is said that at basic pH levels thiosulphinates undergo hydrolysis to form disulphides (Kice & Rogers, 1974; Müller, 1989), which may be important in the latter regions of the intestinal tract where the pH of the environment is more alkaline. However in terms of undergoing metabolic transformations in body tissues, it has been shown that allicin is converted to DADS during passage through liver tissue (Egen-Schwind, 1991; Pentz, 1991), although it is not known whether this occurs *in vivo.* If this is the case then it will be important to determine whether this occurs in mammalian or microbial cells associated with the intestinal tract. No data has been published on the transformation of sulphides present in G.O

The action of any antibiotic is dependent on the distribution and metabolic activity of the natural flora. *In vitro* it is assumed that all the organisms are at a relatively uniform state of metabolic activity and are equally exposed to the garlic (coming into direct contact) at an essentially constant concentration. In the human body however, there will be a diversity of activity depending on the environmental location and surrounding conditions. The natural flora of the intestinal tract are normally at a low level of biosynthetic activity and therefore may be relatively insusceptible to the action of antimicrobials and these "dormant" organisms may consequently often survive exposure to high concentrations, however the garlic may be unequally distributed within tissues and fluids. The natural microbial flora are often closely associated with mammalian cells and it is known that antimicrobial agents enter the tissue cells at different rates. The absorption of antimicrobials from the intestinal tract is normally irregular (Jawetz *et al*, 1992), as there is a continuous excretion as well as inactivation of the antimicrobial. Consequently the levels of the garlic products in body compartments may fluctuate continually and the microorganisms will be exposed to varying concentrations which may not be significantly high enough to induce inhibition of growth or cell death.

If the hypothesis of incorporation of G.O sulphides into bile salt micelles, allowing the sulphides to become soluble in water (Coleman, 1987) actually takes place then as these micelles are transported down the intestinal tract, a localisation of G.O may occur at micelle absorptive sites of the lining of the intestinal wall producing a high concentration of G.O in an area where attachment of bacteria occurs. It has been shown that garlic powder interferes with the synthesis of adhesin, a protein involved in the adhesion/attachment process of *Candida* species (Ghannoum, 1990). If prevention of attachment can be achieved within the gastrointestinal tract then, since the flow of contents through the intestinal tract is continuous, the retention time of the organism within one particular area would be reduced. However prevention of attachment may also have a detrimental effect

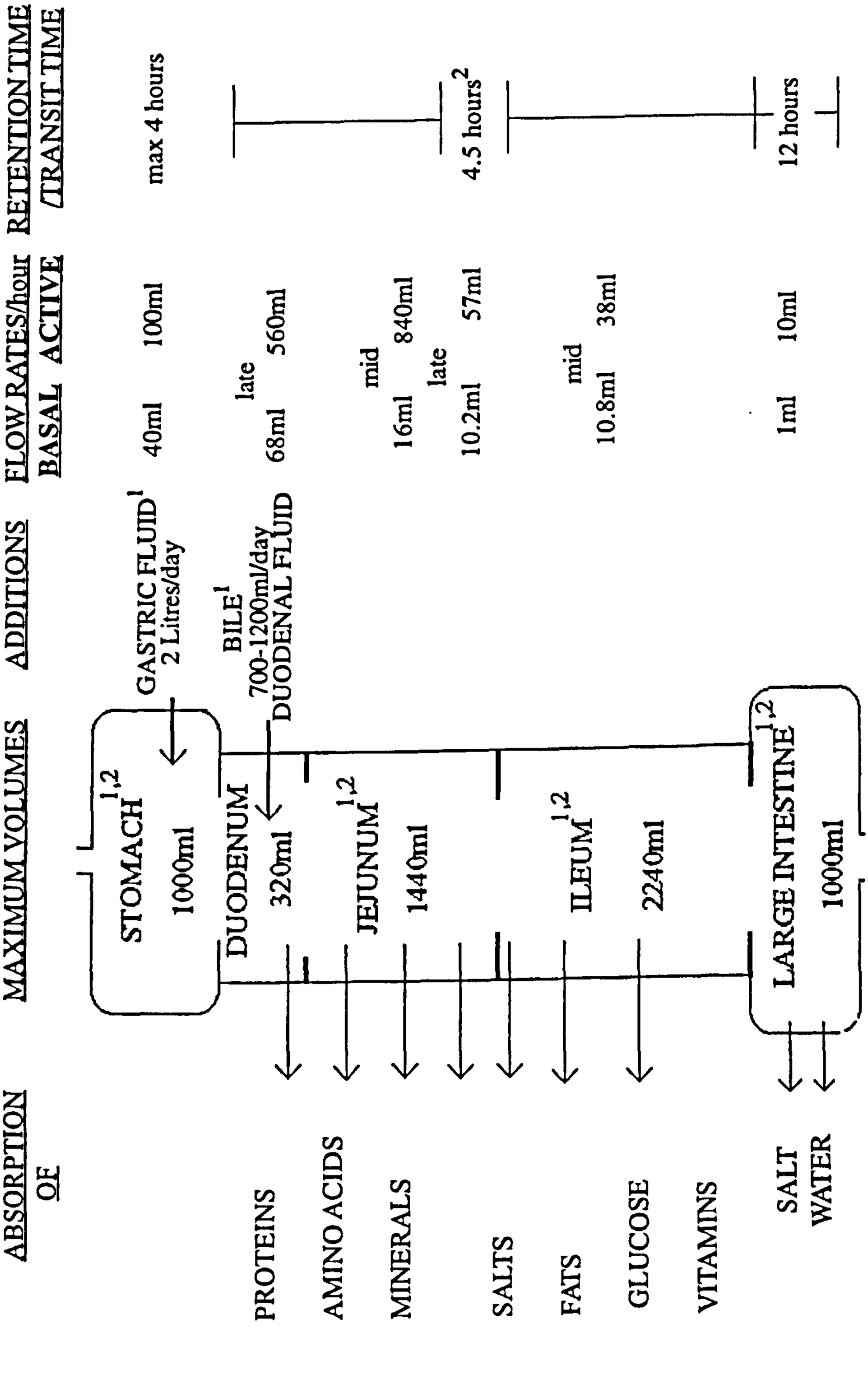
on the natural commensal microflora that are attached to the surface of the intestinal tract.

Infection by foodborne bacteria, such as *L. monocytogenes*, is a chance process dependent upon the number of living infectious bacteria that are present in the intestinal tract, such that, the greater the number of infectious bacteria present, the greater the chance of their attachment to, and invasion of, the intestinal wall lining. The growth and proliferation of natural intestinal bacteria is therefore important as they may reduce risk of infection (as shown in the absence of G.O in ileostomy fluids, Section 3.5.3) due to their antagonistic metabolic capabilities, such as the production of acids, the release of bacteriocins, as well as their role in colonisation of the intestinal wall linings which reduces the chance of attachment by infectious bacteria (Nelson & Mata, 1970; Savage, 1972). Therefore the effective population size of the infectious bacteria is important in determining the mode of infection.

An important outcome of this work was to determine an antimicrobially effective oral dose (against at least some pathogens) of a chosen garlic product and its effect on the natural microorganisms as well as the incoming foodborne pathogens. In ileostomy fluid (IF) the effective concentration of G.O required to reduce but not eliminate the *L. monocytogenes* population whilst allowing the natural flora to remain at a stable population size was approximately 0.34mg/ml. This compares with MIC results obtained in TSB (Section 3.1), which indicated that for G.P and G.O, concentrations of 6.25 and 0.02mg/ml (and higher) respectively are required to inhibit the growth of this bacterium. To establish whether such inhibitory and lethal effects can be achieved *in vivo* requires further *in vivo* human or animal studies (See Future Work). One of the most important aspects to consider is the achievement of sufficient garlic product concentrations; it is critical to maintain an effective concentration of the garlic product at locations where the infecting organisms survive or proliferate for a sufficient length of time to eradicate them.

Many factors within the GI tract will have an influence on the final concentration of garlic product reaching the site of infection. These influencing factors in the GI tract are represented in Figure 4.6.2a.

Figure 4.6.2a: Factors Affecting The Distribution Of Garlic Products In The GI Tract



Reference Source: 1, Sanford, P.A. (1992)
2, Kutachi, H.C. (1988)

The processes of secretion, absorbtion and bulk transit in the different areas of the small and large intestine will all influence garlic product levels. This is due to the fact that the three processes identified all vary in rate immensely and the fluid volumes of different parts of the intestine also vary to correspondingly great extents. The variations in the three process referred to primarily result from the varied size, composition and rate of ingestion of food. Estimates of quantitative garlic molecule concentration are further made difficult by the limited data available on most of the alimentary processes in different parts of the gut. Thus although broad ranges for the rates of secretion of the major intestinal structures are available, little is known about the rates of absorbtion except that overall these match those of secretion. Of value in this connection however is the knowledge that gastric chyme amounts to around 7 litres/day of which 5½ litres is reabsorbed by the end of the terminal ileum (Sanford, 1992). Also the location of absorbtion is throughout the intestine (though mainly jejunal) for water and to some degree all water soluble substances, whereas most hydrophobic substances (such as lipids) and therefore perhaps the hydrophobic garlic sulphides also are absorbed mainly in the lower region of the small intestine. However no specific information concerning the site of absorbtion of garlic molecules is available. Pentz *et al.* (1991) have shown that both the precursor of allicin (alliin) and the main garlic sulphide (DADS) are rapidly metabolised to unknown secondary products by small intestine mucus membranes *in vitro.* It is possible therefore that most or all the regions of the small intestine tissue may readily absorb allicin and sulphides.

By combining assumptions about meal sizes with information of the type referred to above various digestion-absorbtion scenarios may be constructed. These have been performed so as to allow approximate estimates of plausible concentrations of garlic product molecules to be made.

i) Brief Early Meal Period (such as/or a snack)

An average intake of food during a meal is approximately 500ml/hr, therefore following fasting within about 10-15 minutes 75-125ml (100ml) of food would enter the duodenum and mix with approximately 100ml of secreta (Kutachi, 1988; Sanford, 1992; Pietroni, Personal Communication). Little absorbtion is likely during the first 10-15 minutes of a meal and is ignored as a factor. Thus, consumption of 1 Höfels Cardiomax G.O perle (the maximum dosage perles on the market, containing 4mg of G.O) along with a meal would provide a concentration of G.O in the upper/mid jejunum of 0.02mg/ml. By comparison consumption of 1 Kwai garlic powder tablet which is the highest dose garlic powder tablet (100mg) on the market would provide a G.P concentration in the upper/mid jejunum of 0.5mg/ml.

If garlic molecule absorbtion is terminal ileal, concentrations of these amounts (multiplied by a factor of 7.0/1.5) might be obtained in the mid-ileum. Thus for G.O and G.P concentrations of 0.09 and 2.3mg/ml respectively would be provided.

ii) After A Complete Meal

If the garlic products are consumed after a meal and mixes thoroughly within the stomach (assuming 500ml of food is diluted with 560ml of secreta), assuming no absorbtion takes place, by the time the mid/upper jejunum is reached a concentration of 3.7µg/ml (G.O) and 0.093mg/ml (G.P) would be provided.

If however progressive absorbtion of the garlic products occurs then perhaps half this level would be present (1.85µg/ml (G.O) and 0.046mg/ml (G.P)). If however terminal ileal absorbtion of the garlic products takes places the concentrations are again multiplied by a factor of 7.0/1.5, therefore providing G.O and G.P concentrations of 0.017 and 0.433mg/ml respectively.

iii) During Fasting

If the subject is fasted, one would assume a quicker transit of G.O through the intestinal tract with a lower dilution according to the basal flow rates (approximately

68ml secreta/hour), assuming minimal absorbtion under these condition the concentration of G.O and G.P in the upper/mid jejunum would be 0.06mg/ml and 1.46mg/ml respectively.

Assuming these projections are correct comparisons of the garlic molecule concentrations to MIC determinations (Section 3.1) can be made (Table 3.6.2b, p262). The table indicates inhibition of growth of a range of (non- and pathogenic) organisms can be achieved by the predicted concentrations of the garlic products (determined above). On the basis of these calculations it could be assumed that amounts of calculated G.O but not G.P would produce potentially useful inhibition of antimicrobial activity against growth of several of the pathogenic bacteria screened in Section 3.1, if consumed at the beginning (or after fasting) rather than after a meal.

To obtain in the intestine the 40µg/ml (or higher) G.O or 6-25mg/ml G.P concentrations determined by MIC determinations would require consumption of 2 or more G.O perles (3 or more G.P tablets), if consumed at the beginning of the meal. If consumed after the meal 10 or more G.O perles (15 or more G.P tablets) would be required. These doses would be safe and acceptable for the acute short-term treatment of "tummy" upsets. Using the viability study data in CIF obtained in Section 3.5.1 and 3.5.1.1, to obtain kill of the following organisms; *E. coli* (40) 2.75mg/ml, *L. monocytogenes* (433) 0.68mg/ml, *S. typhimurium* (434) 2.75mg/ml and *Shig. sonnei* (426) 0.34mg/ml would require unrealistic quantities of garlic oil perles such as 137.5, 34, 137.5 and 17 capsules if consumed along with a meal. However much smaller doses taken over a period of a few days might prove biocidal.

Table 3.6.2b: Relation Of Projected Garlic Product Concentrations To The Inhibitory Concentrations Against Enteric Non- And Pathogenic Bacteria

* denotes enteric pathogens

Projected Garlic Concentrations	Organisms (Culture No.)	MIC (24 hours) mg/ml
Brief Early Meal		
G.O 0.02-0.093mg/ml	*Bacteroides fragilis* (333a, b) *Klebsiella aerogenes* (325) * *L. monocytogenes* (433a, b) * *Vibrio metshnikon* (437) * *Yersinia enterocolytica*	0.03 0.04 0.02 0.02 0.01
G.P 0.5-2.3mg/ml	No Organisms	
After A Complete Meal		
G.O 1.85-17µg/ml	No Organisms	
G.P 0.046-0.433mg/ml	No Organisms	
During Fasting		
G.O 0.06mg/ml	*Bacteroides fragilis* (333a, b) *Klebsiella aerogenes* (325) * *L. monocytogenes* (433a, b) * *Vibrio metshnikon* (437) * *Yersinia enterocolytica*	0.03 0.04 0.02 0.02 0.01
G.P 1.46mg/ml	No Organisms	

It should be noted that the garlic product doses obtainable and the bacterial sensitivities to them referred to are inevitably tentative. Amongst the particular qualifications are the; 1) uncertainty about the duration of suggested garlic molecule concentrations in particular gut locations and its related antimicrobial contact; 2) possible metabolic conversion of garlic molecules to other more or less antimicrobials; 3) possible occurrence and extent of resecretion of absorbed garlic molecules or their metabolic products AND 4) impact upon antimicrobial effect or possible garlic molecule actions upon bacterial motility and attachment. In conclusion, the

achievement of bacteriostatic and perhaps bacteriocidal concentrations of garlic products intestinally appear realistic possibilities in the remedy of at least some bacterial pathogens indicative of enteric infections. In addition lower doses of garlic products taken regularly may benefit in assisting the human body to prevent the establishment of gastrointestinal infections.

CHAPTER 5

FUTURE WORK

5.0 Future Work

Garlic oil and garlic powder have both been shown to be antimicrobial. The oral dose required to inhibit the growth of pathogens intestinally, based on *in vitro* batch culture experiments performed in this study and using theoretically determined values for dilution effects during passage into the small intestine has been deduced. The values obtained are probably not too high a concentration to be realistically acceptable by the general population in acute infections requiring 1-2 days treatment only. The main aim of this study was: Could garlic-derived products have realistiic potential in the control or elimination of enteric pathogens involved in food poisoning at **acceptable realistic oral doses?** To some extent this was answered by the results obtained, however further work is required to fully confirm this with more accurate modelling systems and by *in vivo* trials. The second aim, in determining the mechanism of garlic action was also studied.

The possibility that underestimation of the relative potency of G.O due to loss of essential components by volatilisation was realised in this thesis and in attempt to prevent this loss a variety of cap sealing methods were investigated. It was identified that tight-fitting caps reduced volatilisation of G.O and hence additional work could attempt to further reduce the extent of volatilisation by using gas-tight containers (such as screw-tight septum capped tubes). This may however limit further antimicrobial studies to anaerobic/microaerophilc organisms only unless maintaining respiratory gas exchange can be overcome.

The individual sulphides present in G.O have been shown to be antimicrobial however none of those studied could fully account for the antimicrobial effectiveness of G.O, suggesting that the antimicrobial activity may be due to the presence of other components, not studied in this project, having a greater "effectiveness" or that there are synergistic effects between two or more components. Alternatively as discussed (p215) the differences detected may be partially or whole artefactual

due to possible differences in volatilisation rates which could be reduced by the introduction of gas-tight vessels as described above. Irrespective of whether the differences between G.O and individual garlic sulphides are real or not, further studies are required into the antimicrobial effectiveness of the individual components of G.O. Initially such a study would require purification of the individual sulphide components of G.O as there is limited commercial availability of G.O sulphide component preparations. Purification could be achieved by involving preparative Medium Pressure Liquid Chromatography (MPLC) using a suitable volume of G.O, the different fractions could be collected then concentrated and identified. Purification checks on the individual sulphide sample fractions would then need to be made (by HPLC) before MIC determinations can be performed. The synergistic hypothesis could be studied by comparing the activities of individual sulphides with those of sulphide combinations in the same proportions present in G.O to that of fresh G.O. A consequence of such a study may be the identification of one or more G.O components which are antimicrobially more effective (in terms of inhibition of growth rates of the pathogens) at lower concentrations than that of the determined G.O MIC values and hence as a purified product could facilitate realistic oral doses. In terms of garlic product analysis further work is required to study the conversion of thiosulphinates to other components (including sulphides) especially in the presence of microbial cells (*in vitro*) as well as possible conversions *in vivo*. This is of importance as it may provide an explanation of the relative "lag"/ stationary phases of growth of bacteria in the presence of G.P not observed with G.O.

It is possible that concentrations of G.O or garlic components lower than their respective MIC values may be more effective than those determined in the Discussion Section if; 1) a reduction in growth rate of the pathogen *in vivo* facilitates the natural host defences sufficient to overcome and eliminate the pathogen AND 2) are effective against other pathogenic determinants required for disease to occur

such as motility, required by cells (such as *Campylobacter spp* and *Salmonella spp*) to reach attachment sites, inhibition of attachment of the pathogen to the intestinal wall; and/or inhibition of toxin production. These effects on pathogenic determinants involve extracellular microbial proteins which may be susceptible to configurational changes (hence a loss of function) at garlic concentrations lower than that required to inhibit growth (See Section 3.8).

In vitro studies (in this thesis) performed with both synthetic gut fluids and ileostomy effluents have provided information on the sensitivity of bacterial strains to G.O within these environments. These experiments do not take into account the other factors involved, such as, kinetics of the emptying of the stomach, gastric secretion (pH), bile concentrations or intestinal transit rates. All these factors play an important role in terms of any dilution factors involved during the passage of the garlic products down the intestinal tract and the effects of garlic products within the intestinal tract. Thus, the next step would be to develop more complex *in vitro* model systems. Initially this would require further studies using mixed culture batch experiments and subsequently the employment of continuous-flow models of the intestinal tract to include periodic input of garlic materials. A number of well developed continuous-flow model systems of the gastrointestinal tract have been published and could be employed such as a computer controlled *in vitro* model of the gastrointestinal tract (Minekus *et al*, 1993) or the use of a multi-chamber (SHIME) reactor (Molly *et al*, 1993). These complex model systems comprise of a number of successive reactor units simulating the stomach, small and large intestines. A number of other reactors are added for the addition of food and the collection of effluent waste. In order to obtain a bacterial situation similar to that of "normal" human intestinal ecosystems, the reactors are inoculated by adding faecal suspensions. These are more complex model systems than those used in this thesis, since they model the whole intestinal tract on a continuous-flow basis and have shown to yield data comparable to that determined in various areas of the

digestive systems of humans (Savage, 1977; Cummings & Macfarlane, 1991; Macfarlane *et al*, 1992). The value of such intestinal simulators will however be limited due to the absence of absorption of metabolites and fluid factors which may influence G.O concentration *in vivo*. However, although a variety of additives could easily be administered such as bile salts and antimicrobial agents (including garlic) and therefore these methods may provide a more suitable situation in which to assess the effect of the garlic products on the residence time of potential pathogens within the intestinal tract. One of the main drawbacks of these types of system is that they do not accurately model situations in which the respective pathogen maintains itself intestinally by the process of attachment to the intestinal lining, however this has already been overcome with synthetic linings of the reactor vessels allowing attachment to take place. In addition the detection of any effect on the intestinal bacterial numbers would be limited by the available selective media (as stated previously) therefore, instead of determining bacterial numbers these systems are best evaluated by monitoring microbial activity in terms of fermentation fluxes and products, such as, volatile fatty acids, enzyme activities (Humble *et al*, 1977; Molly, Personal Communication). However with the advancement of diagnostic techniques and improvements in isolation media, individual bacterial numbers could be determined.

Thus these model systems have limitations and may not accurately model real situations. The gastrointestinal tract is a constantly changing environment and the relatively poor knowledge and understanding of the microbial ecology of the tract and mechanisms of pathogenicity makes it particularly difficult to model. In addition it is also difficult to predict oral doses of garlic products required to achieve effective concentrations *in situ* within the intestinal tract. A number of clinical trials would therefore be required to establish; 1) relationships between the concentration of the regular oral doses of garlic products to that concentration achieved in the various regions of the intestinal tract AND 2) the effect of regular oral dosing on

intestinal flora of the small intestine and its effect on the fate of pathogens.

No modern clinical studies into the antibiotic effects of garlic or its manufactured products have been performed (Fulder, 1990). The fact that the human intestine is not easily accessible for research purposes and the problems of medical ethics will undoubtedly be the main problems to contend with. It should however be noted that a number of other problems associated with (either intially or during) this work would arise, such as:-

A) a statistically significant number of volunteers willing to take part in a clinical trial.

B) monitoring and control of volunteer's diet - Drasar & Hill (1974) have shown that the human diet (Eastern or Western) will influence the microbial flora of the intestinal tract. Thus it may be necessary to be selective in the choice of volunteers on this basis.

C) administration of the garlic products - this may cause problems both in terms of ethics and safety. The UK product licence for garlic allows the claim of a herbal remedy for the treatment of colds, however each commercial product has a stated recommended dose which may be exceeded in these studies. However results obtained from initial further studies (Section 3.4) may allow for a lower concentration of the garlic components to be used.

D) establishment of a blind placebo trial - this may be difficult as the garlic products are odourous and volunteers taking a placebo will be aware of this fact. This problem may be initially overcome by administering encapsulated garlic products to the test group ensuring release of the components within the stomach. However published data (Rosin *et al*, 1992) has shown that "garlic breath" can still be detected.

E) sufficient quantities of intestinal samples for experimental analyses - this could be achieved with ileostomy effluents, however this would be directly related to problem A) in terms of numbers of volunteers and problems associated with

dissimilarities of the ecology of the gut between these persons and normal healthy individuals (outlined on p233). Endoscopy samples would be the only means of obtaining intestinal samples from healthy volunteers, however it is known that this method provides smaller volumes than those obtained from IF samples. An alternative would be to intially use animal trials which would allow for final sacrifice of the animal.

F) administration of an oral dose of a food poisoning organism will undoubtedly lead to the problems of medical ethics. This may be overcome by the possibility of using attenuated pathogens. Alternatively treatment of patients having contracted food poisoning may be a possibility however this would not be a controlled situation.

In addition to the above laboratory-based *in vitro* studies antimicrobial garlic research of the type performed in this study should also be continued with respect to other clinically important foodborne pathogens, such as *Campylobacter spp*, *Shigella spp*, *Clostridium spp*, with a view to prevention or treatment of infection.

Many intestinal ailments are associated with viral infections and so another area of research could involve determining the effects of garlic products on viruses. Little scientific data is available to support the antiviral properties of garlic especially those involved in gastrointestinal infections. There is however some suggestion that viruses may be more sensitive to garlic than bacterial cells (Weber *et al*, 1992) which may be due to the effect of garlic on exposed viral proteins involved in recognition and attachment. Studies into the antiviral properties of garlic may also provide some interesting data on modes of action.

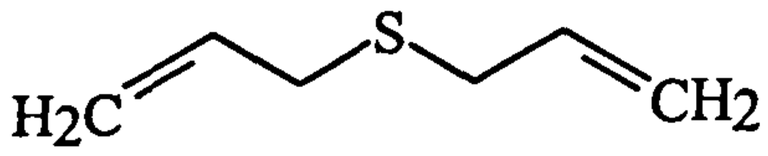
CHAPTER 6

APPENDICES

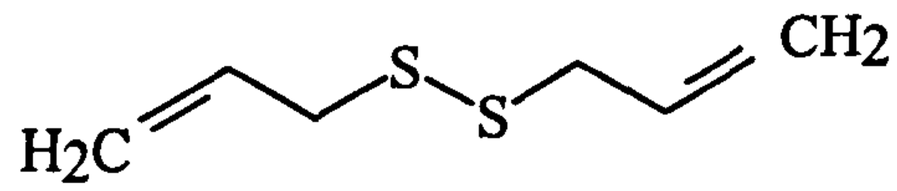
APPENDIX 1

Chemical Structures:

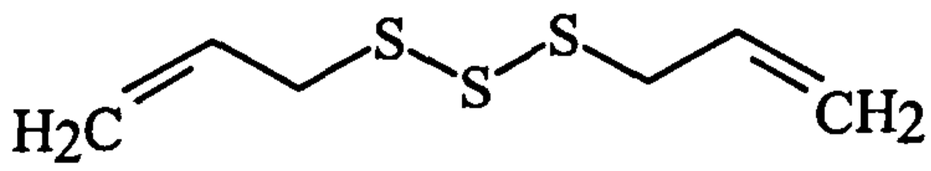
Diallyl sulphide (DAS)



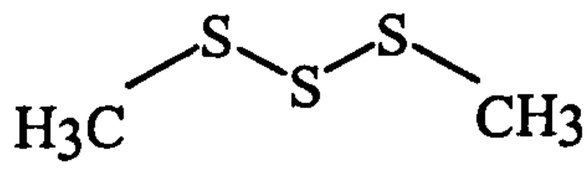
Diallyl disulphide (DADS)



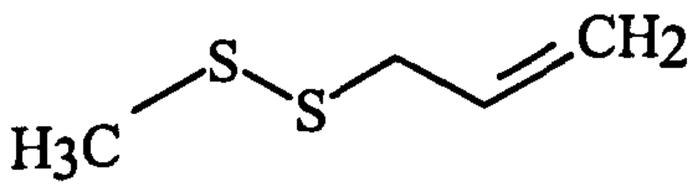
Diallyl trisulphide (DATS)



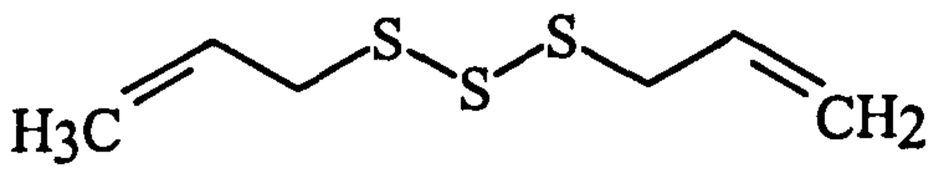
Dimethyl trisulphide (DMT)



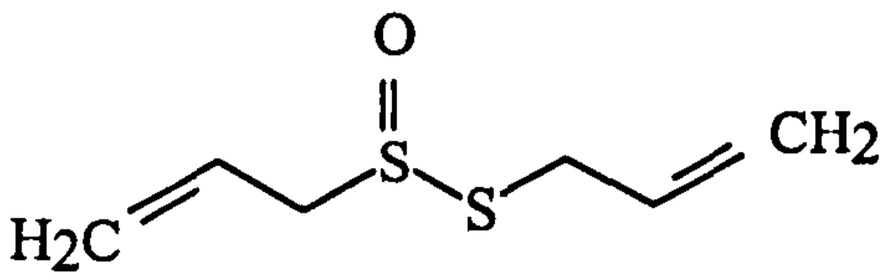
Methyl allyl disulphide (MAD)



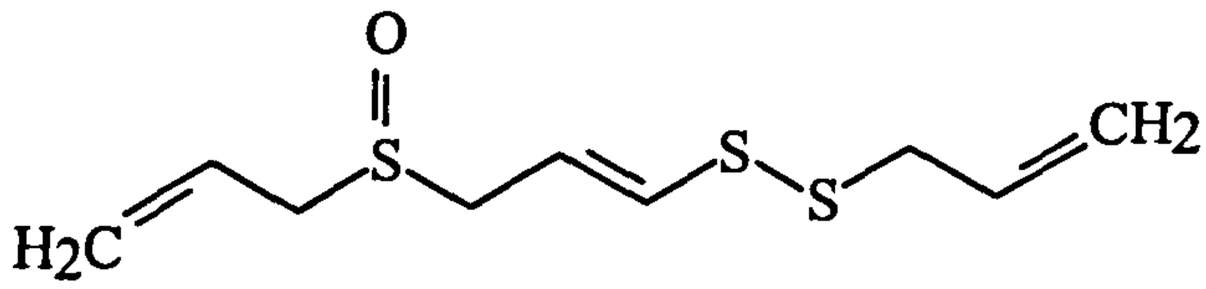
Methyl allyl trisulphide (MATS)



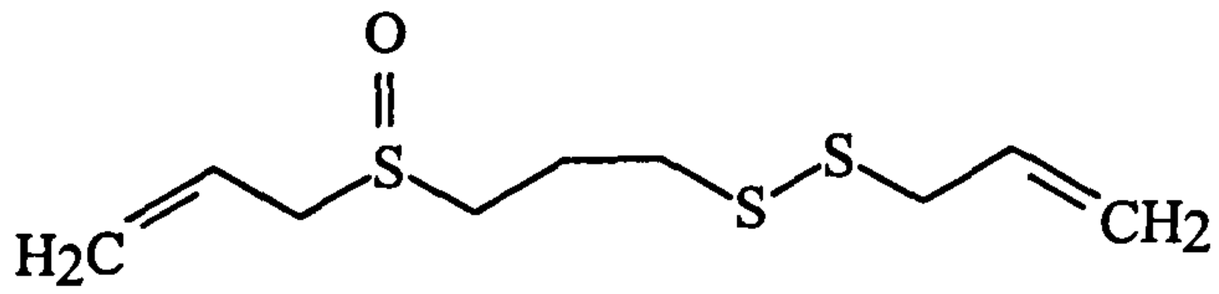
Allicin (diallyl thiosulphinate)



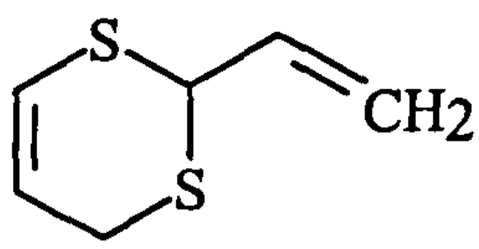
(E)-ajoene



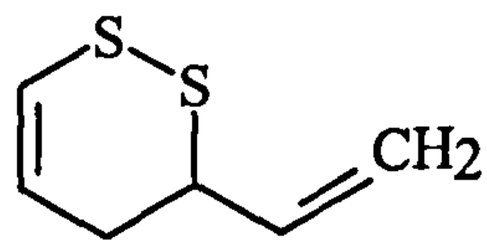
(Z)-ajoene



2-vinyl-4H-1,3 dithiin



3-vinyl-4H-1,2 dithiin



APPENDIX 2

Comparison Of The Potencies Of G.O & G.P Using MIC Values

Data for this comparison was taken from the (replicated) MIC values of G.O and G.P. Strain of bacteria used for calculations: *E.coli* (40), *S. typhimurium* (434), *L. monocytogenes* (433), *B. fragilis* (333).

MIC g/L Broth:	*E. coli*	*S. typhimurium*	*L. monocytogenes*	*B. fragilis*
G.O	0.68	1.37	0.02	0.04
G.P	6.25	6.25	6.25	6.25

	g. Garlic producing 1g. Product	**recommended mg dose/day**
G.O	1600	2
G.P	2.5	800

Specimen Calculations

A. Relative Potency according to Weight of Product

$$\frac{\text{MIC G.P}}{\text{MIC G.O}} = \text{Potency Ratio}$$

e.g For *L. monocytogenes* (333)

$$\frac{6.25\text{g}}{0.02\text{g}} = 312.5$$

therefore, for *L. monocytogenes* (333) G.O is 312.5 times more potent than G.P.

B. Weight of Garlic Bulbs (g. garlic/ml broth) required for MIC value

$$\frac{\text{MIC G.O}}{\text{1000ml Broth}} \text{ X g. Garlic producing 1g. Product}$$

e.g For *L. monocytogenes* (333)

$$\frac{0.02\text{g}}{\text{1000ml broth}} \text{ X } 1600 = 0.032\text{g. Garlic bulb/1000ml broth}$$

(MIC for G.O x g. Garlic/g. G.O)

therefore, for *L. monocytogenes* (333) 0.032g. garlic bulb as G.O provides the MIC.

C. Relative potency according to recommended dose

$$\frac{\text{Recommended dose G.P/mg}}{\text{Recommended dose G.O/mg}} \text{ X } \frac{1}{\text{Relative potency according to wt. of product}}$$

e.g For *L. monocytogenes* (333)

$$\frac{800}{2} \text{ X } \frac{1}{312.5} = 1.28$$

therefore, for *L. monocytogenes* (333), the recommended dose of G.P is 1.28 times more potent than the recommended dose of G.O.

APPENDIX

Plate 1.0 Comparison Of Appearence Of *E. coli* (40) On TSA and LSA plates.

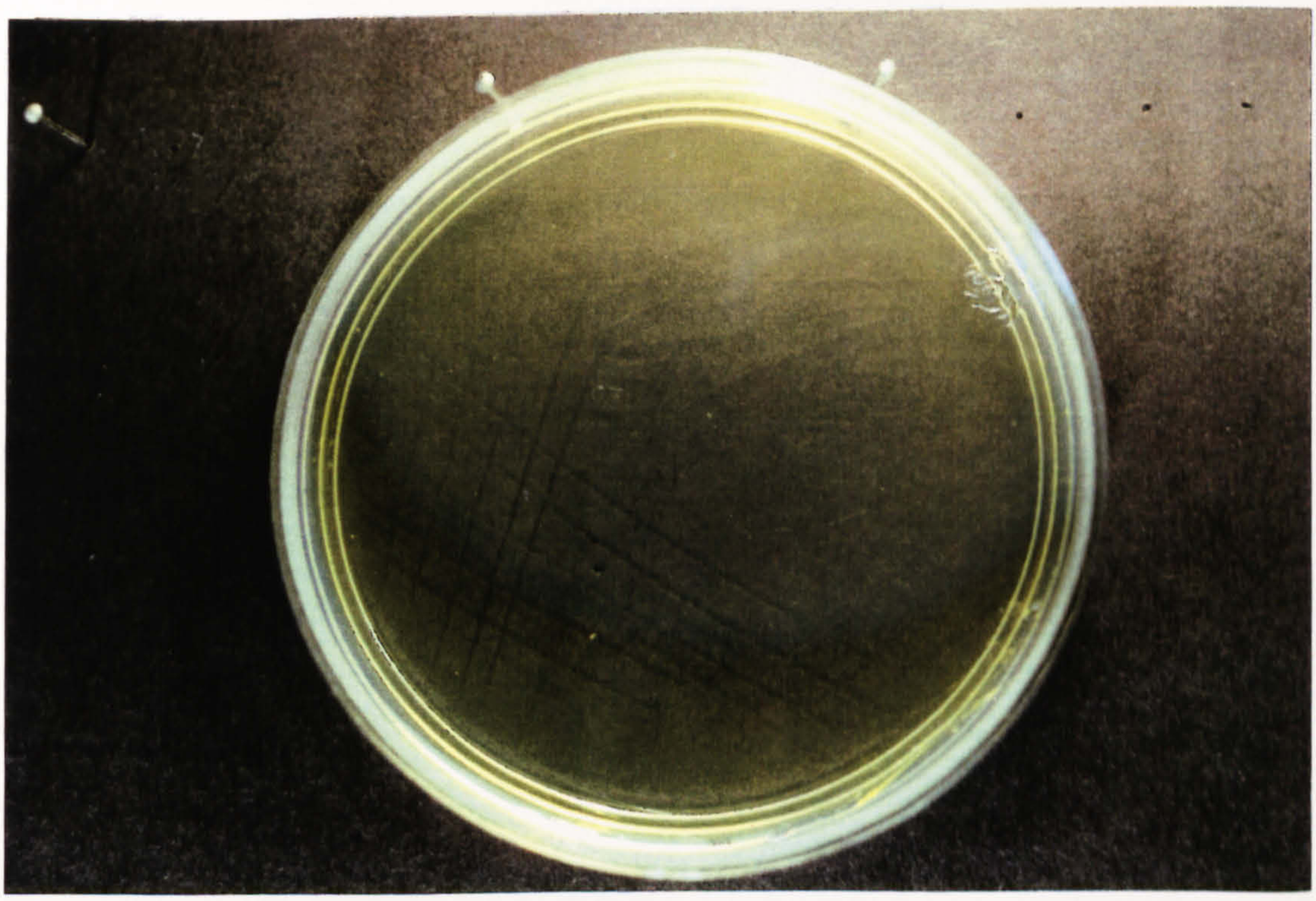


Plate 2.0 Comparison Of Appearence Of *E. coli* (40) On MC and XLD plates.

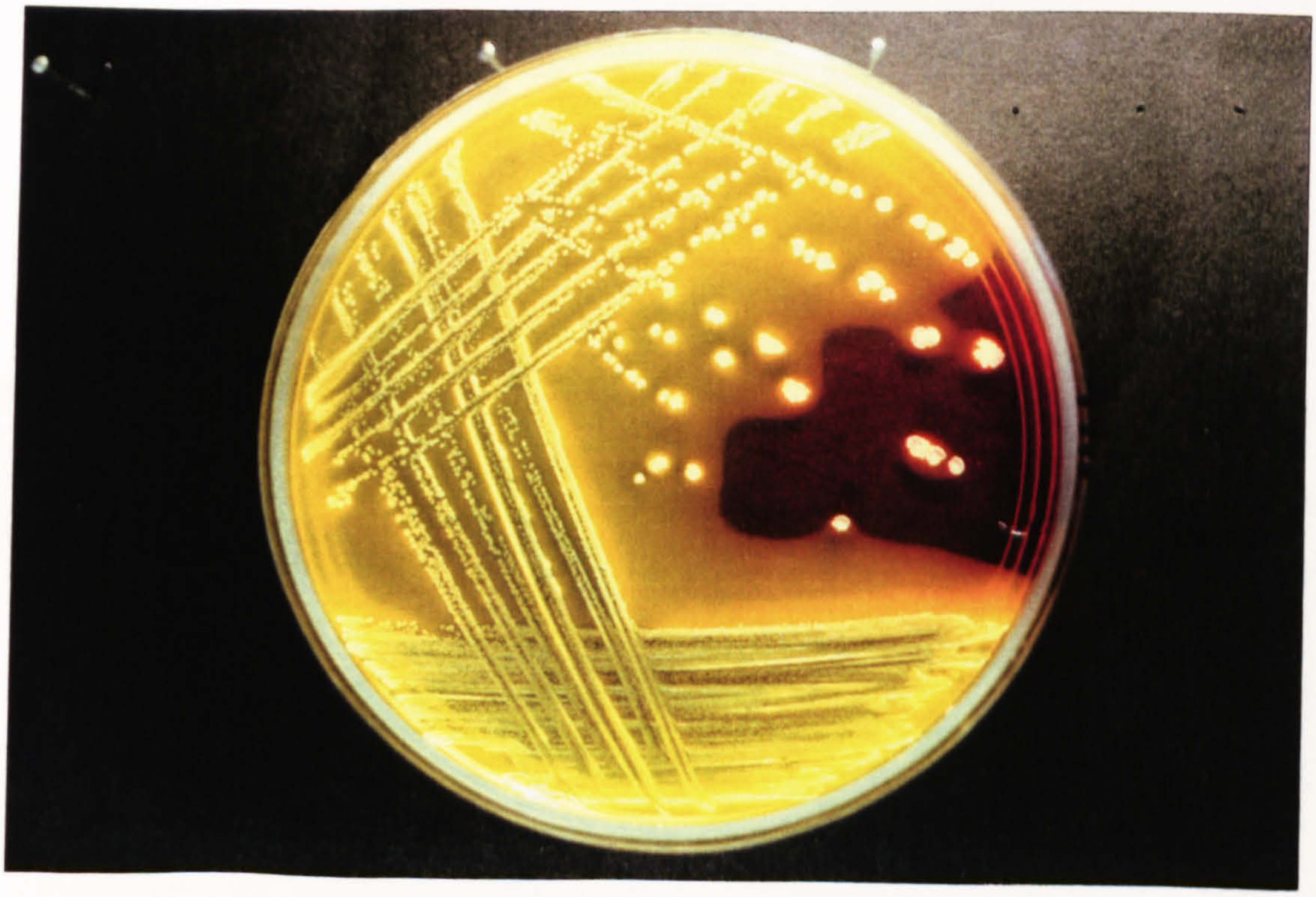
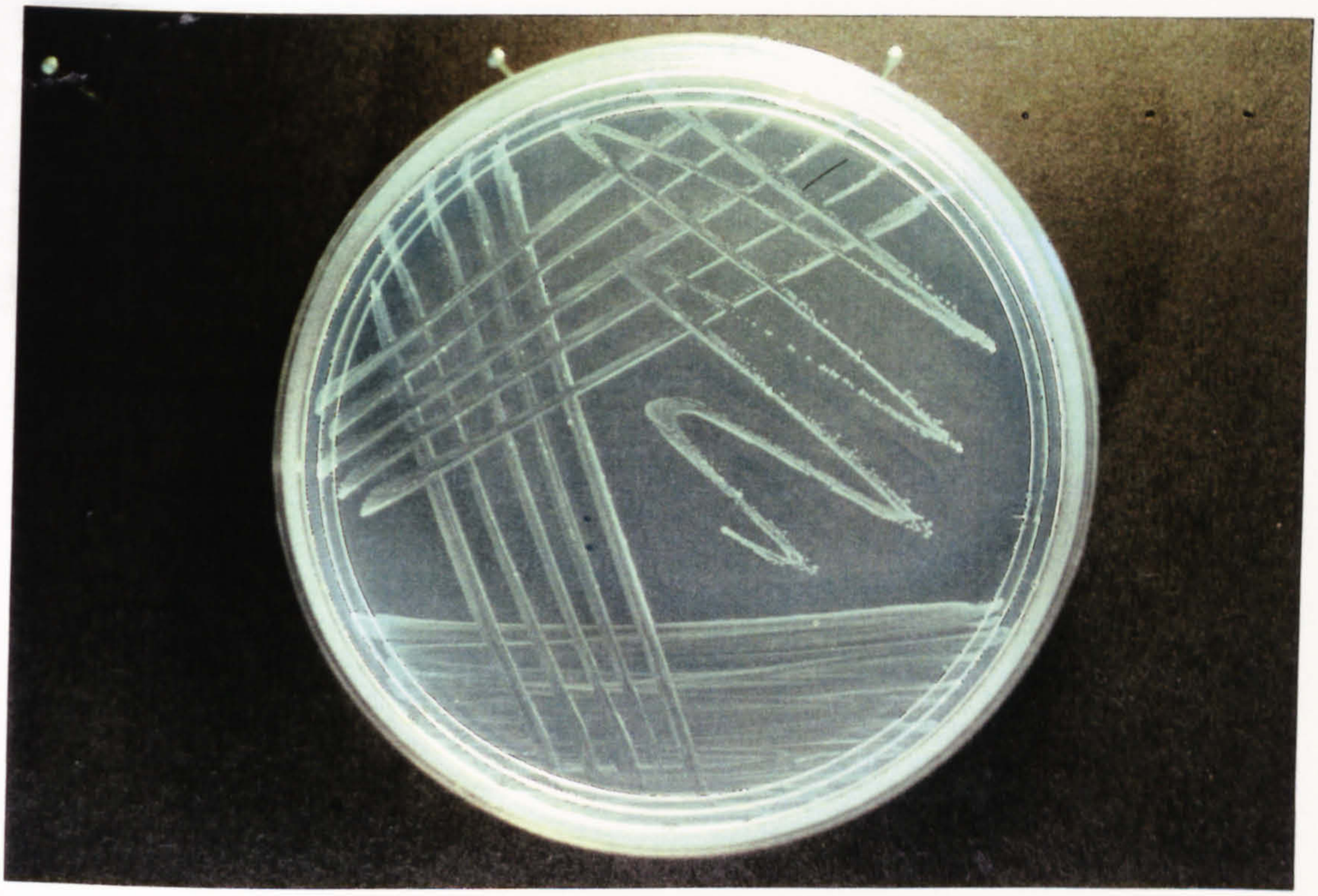


Plate 3.0 Comparison Of Appearence Of *L. monocytogenes* (333) On TSA and LSA plates.



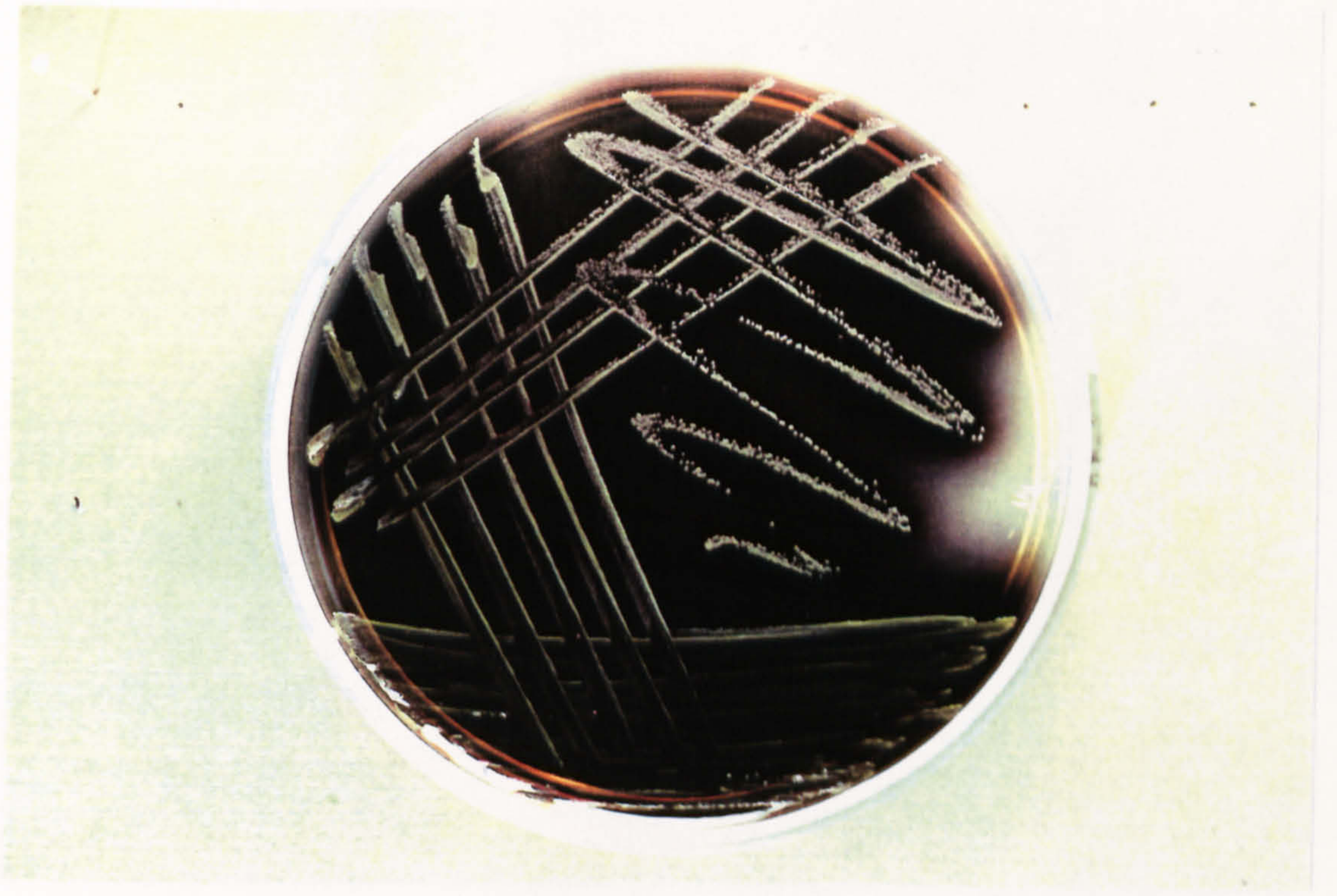
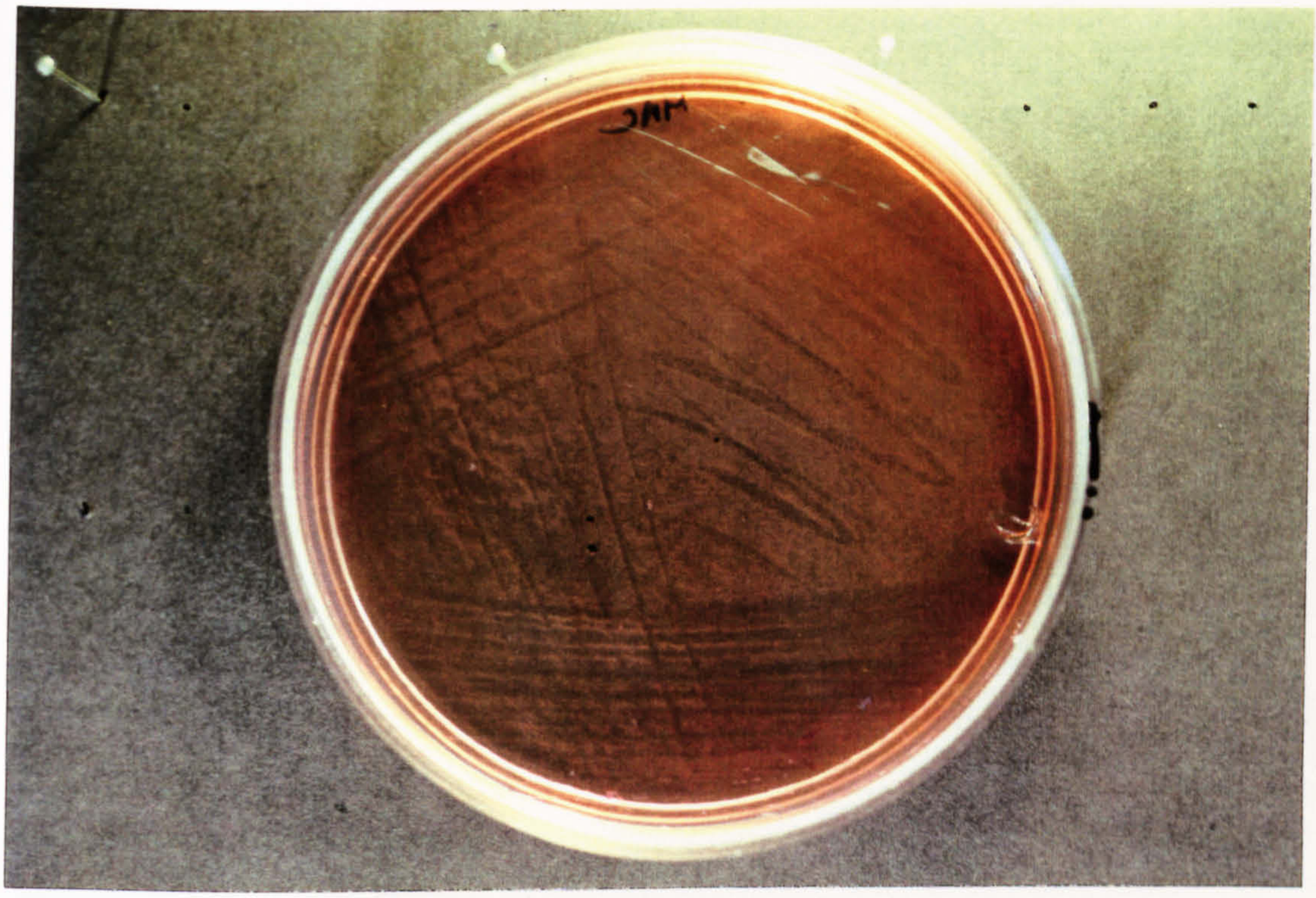


Plate 4.0 Comparison Of Appearence Of *L. monocytogenes* (333) On MC and XLD plates.



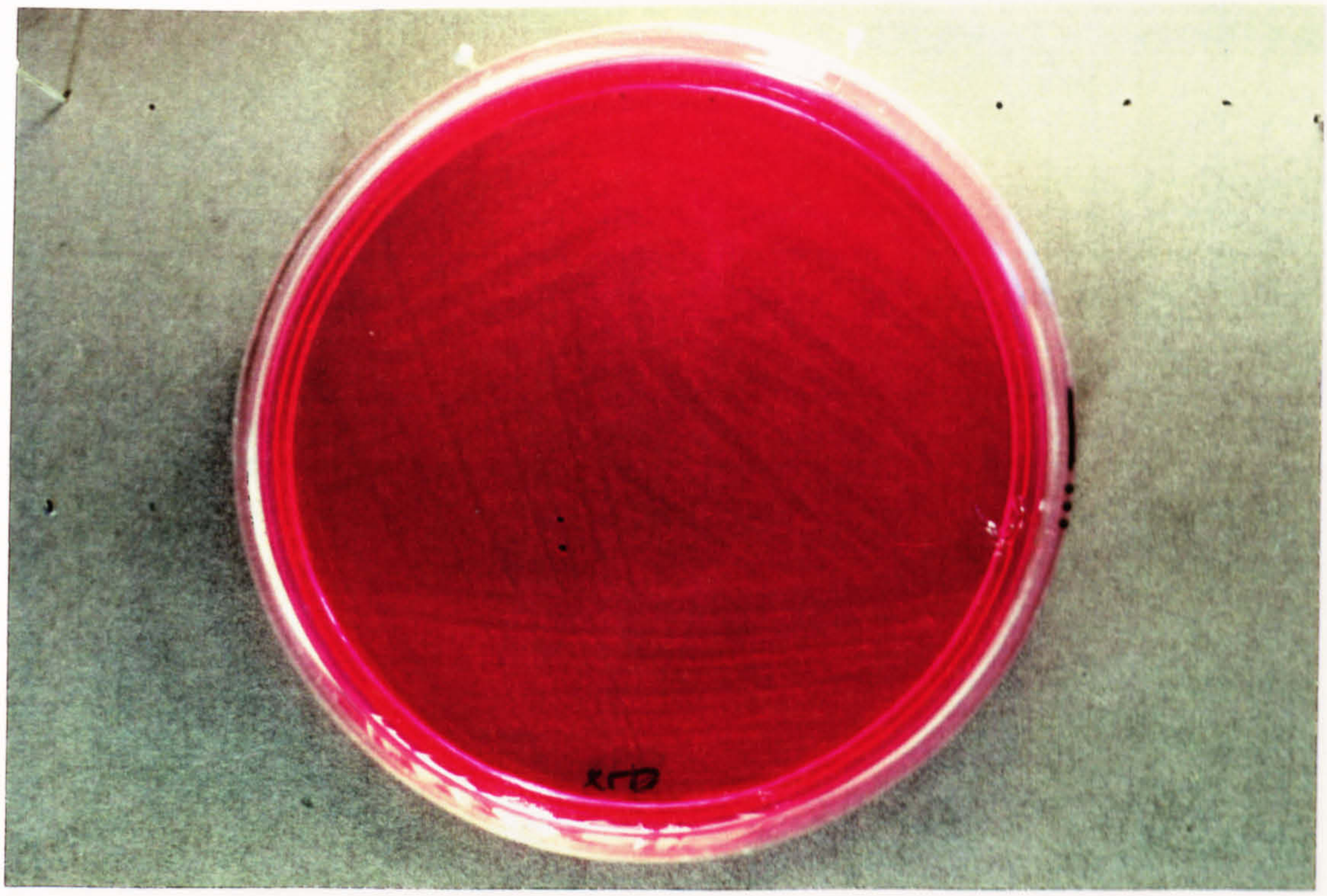
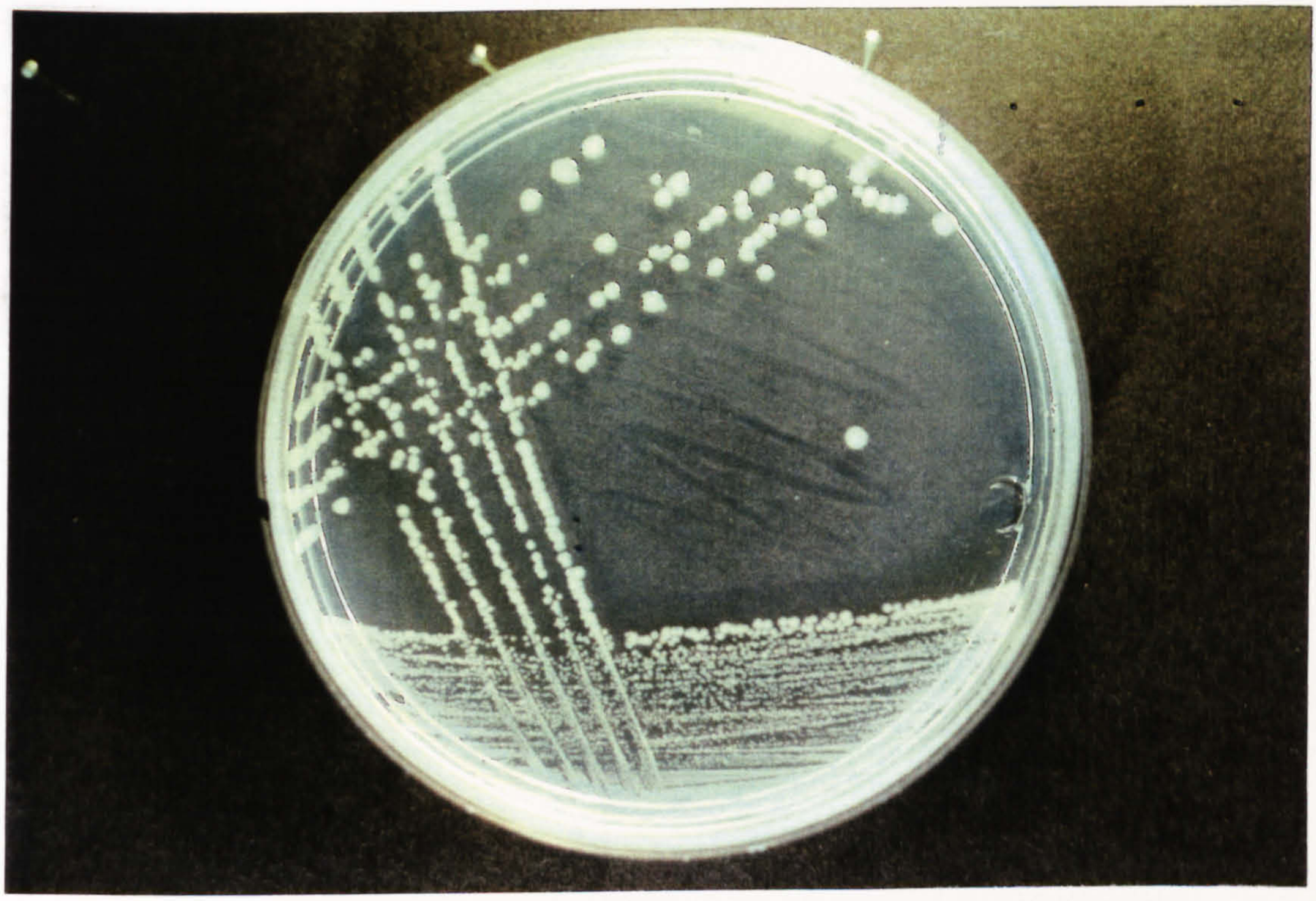


Plate 5.0 Comparison Of Appearence Of *S. typhimurium* (434) On TSA and LSA plates.



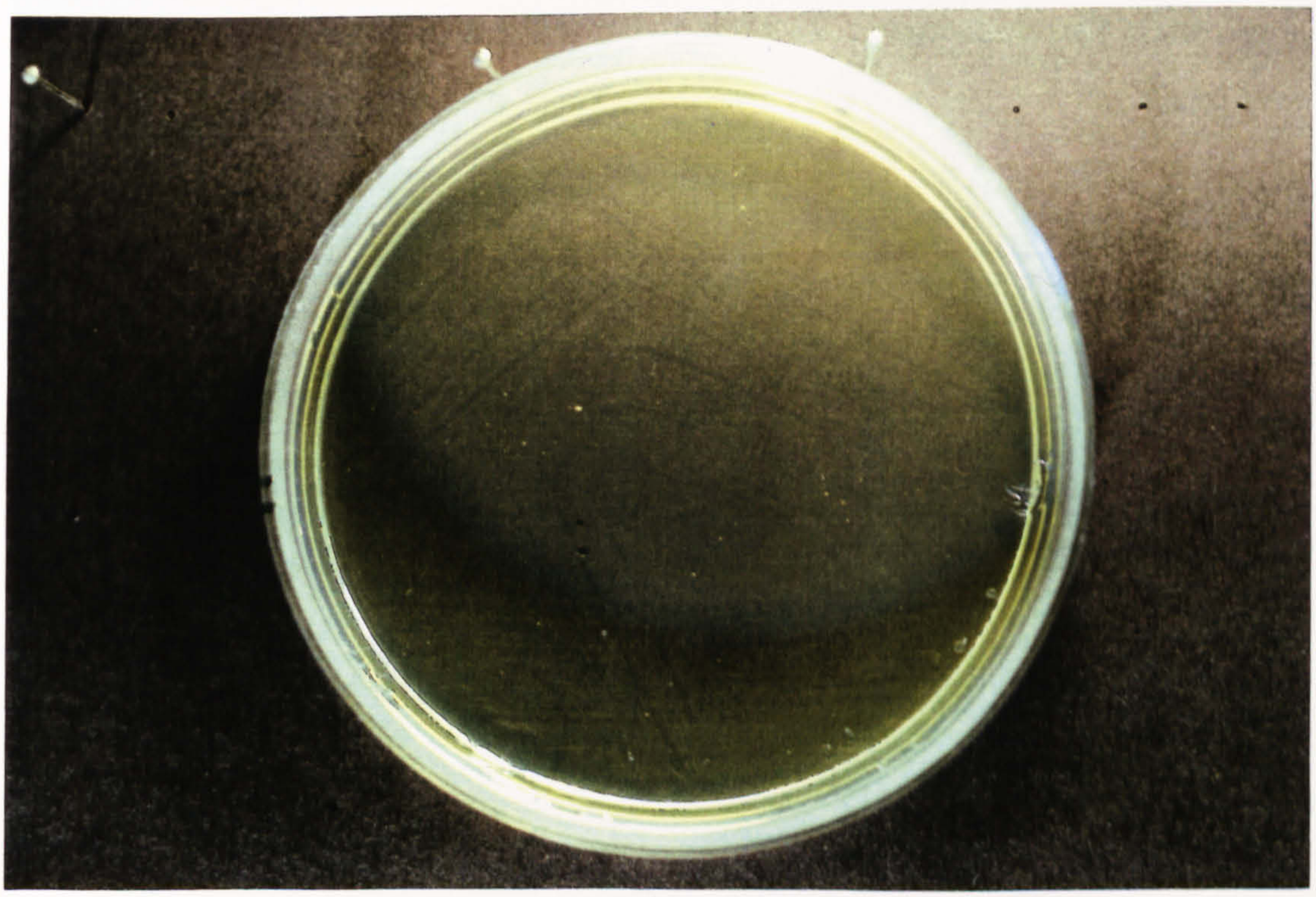
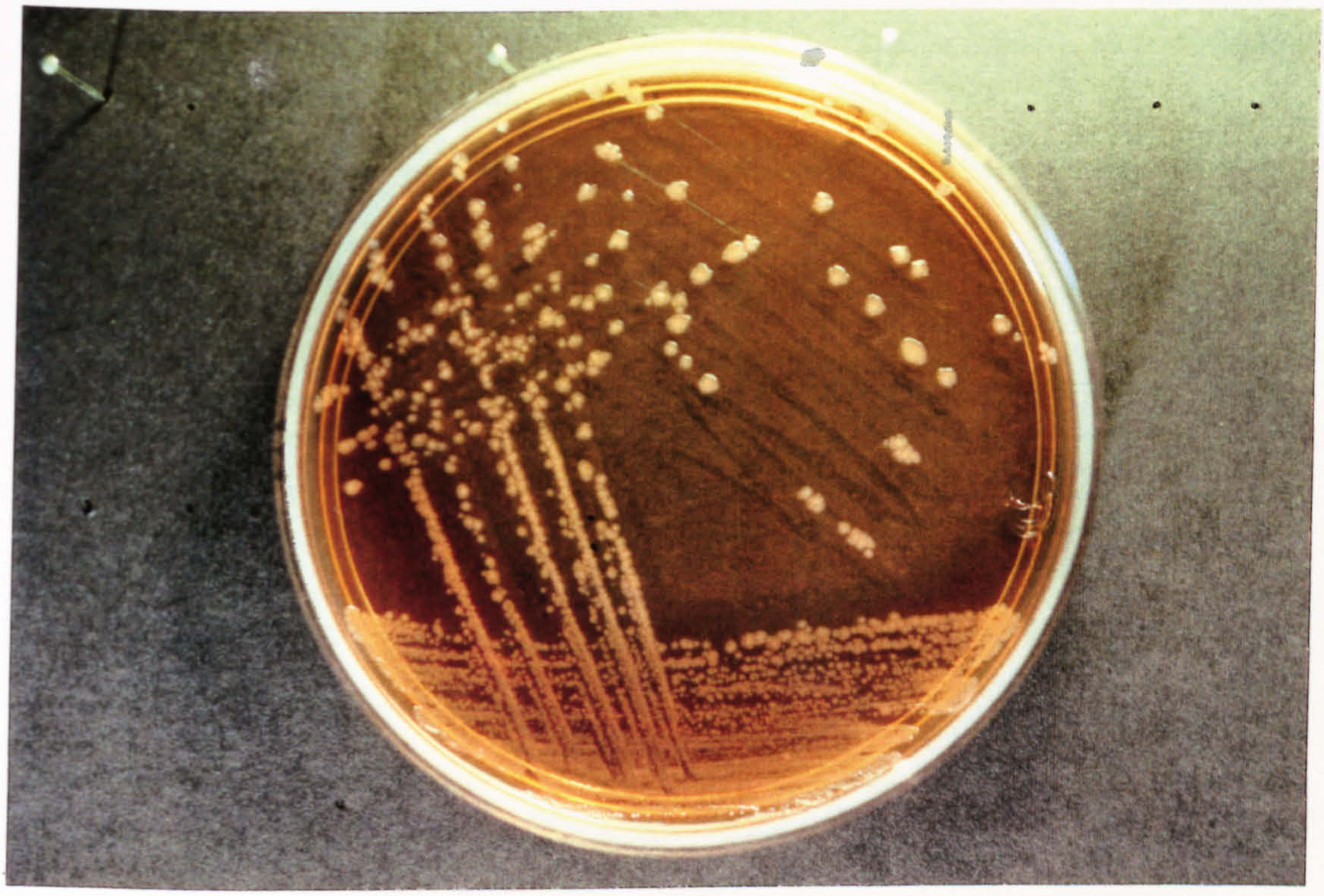


Plate 6.0 Comparison Of Appearence Of *S. typhimurium* (434) On MC and XLD plates.



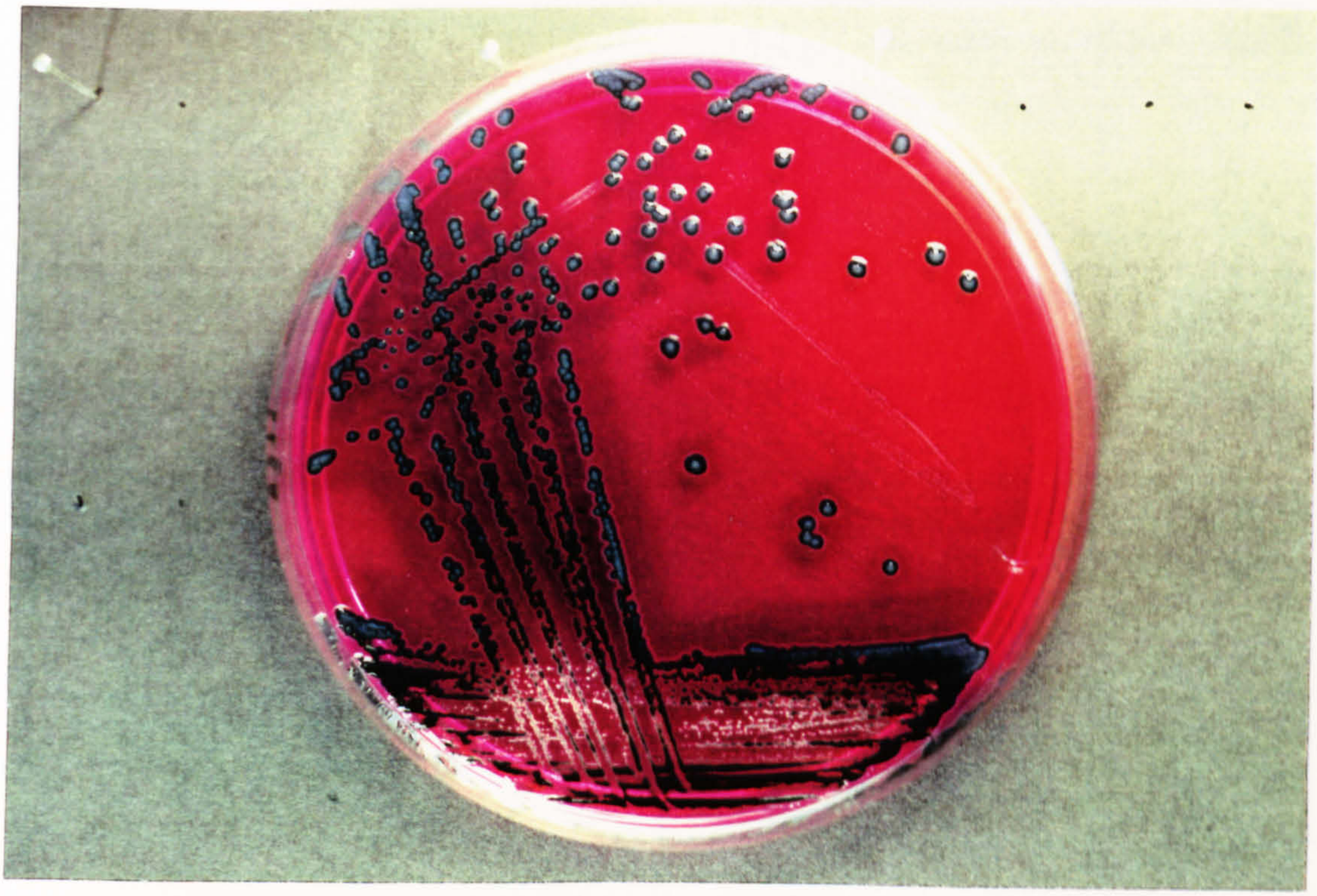
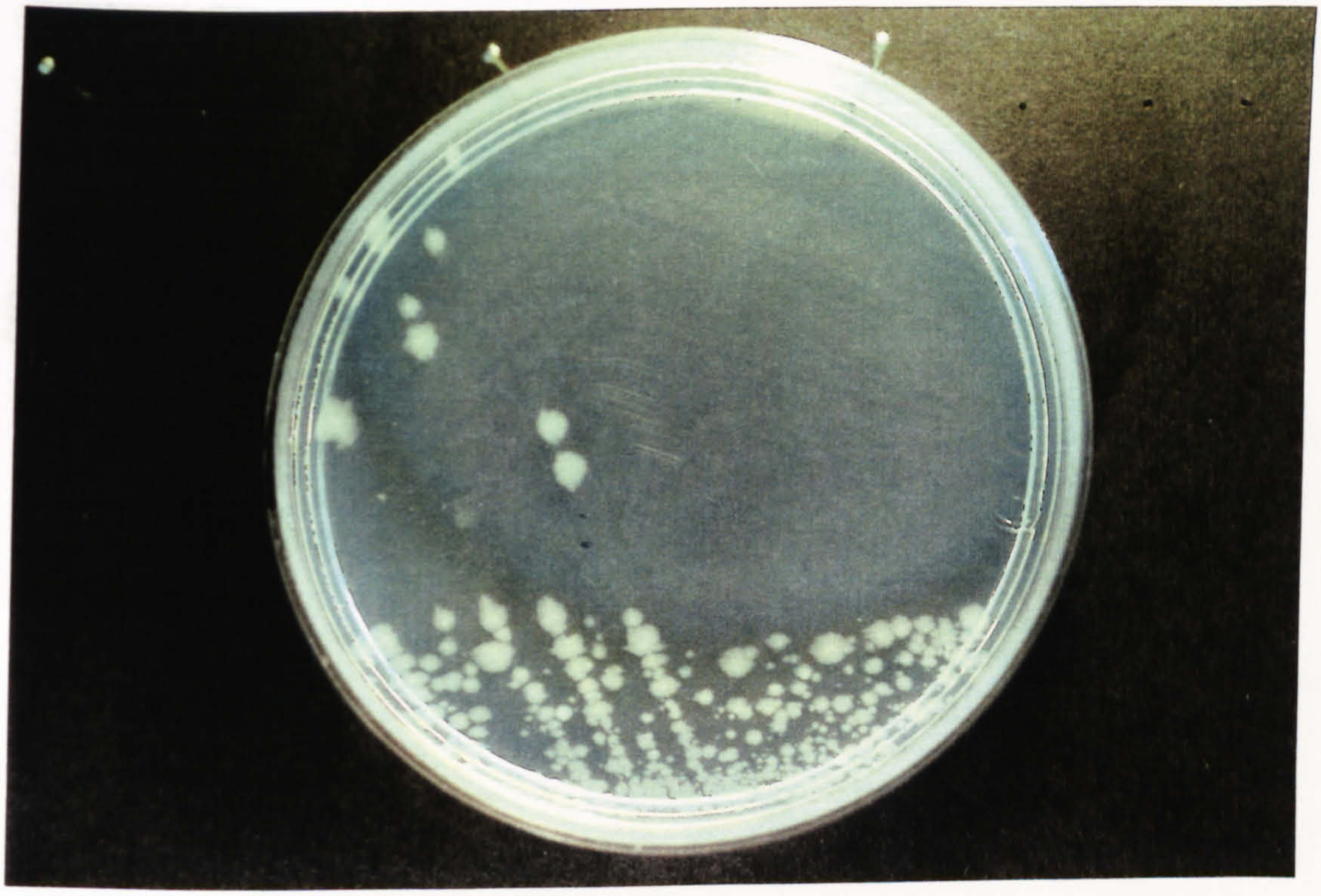


Plate 7.0 Comparison Of Appearence Of *Shig. sonnei* (426) On TSA and LSA plates.



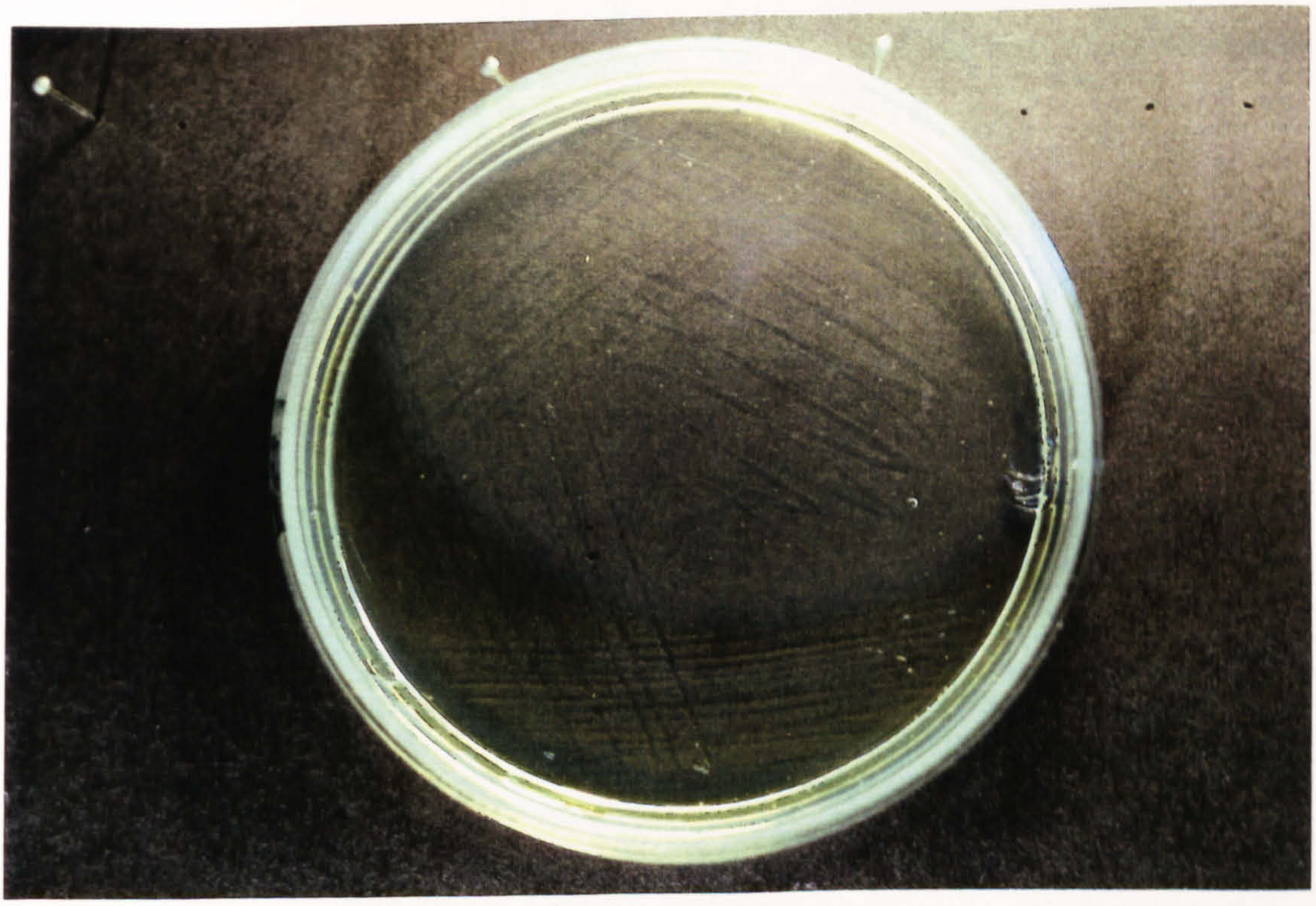
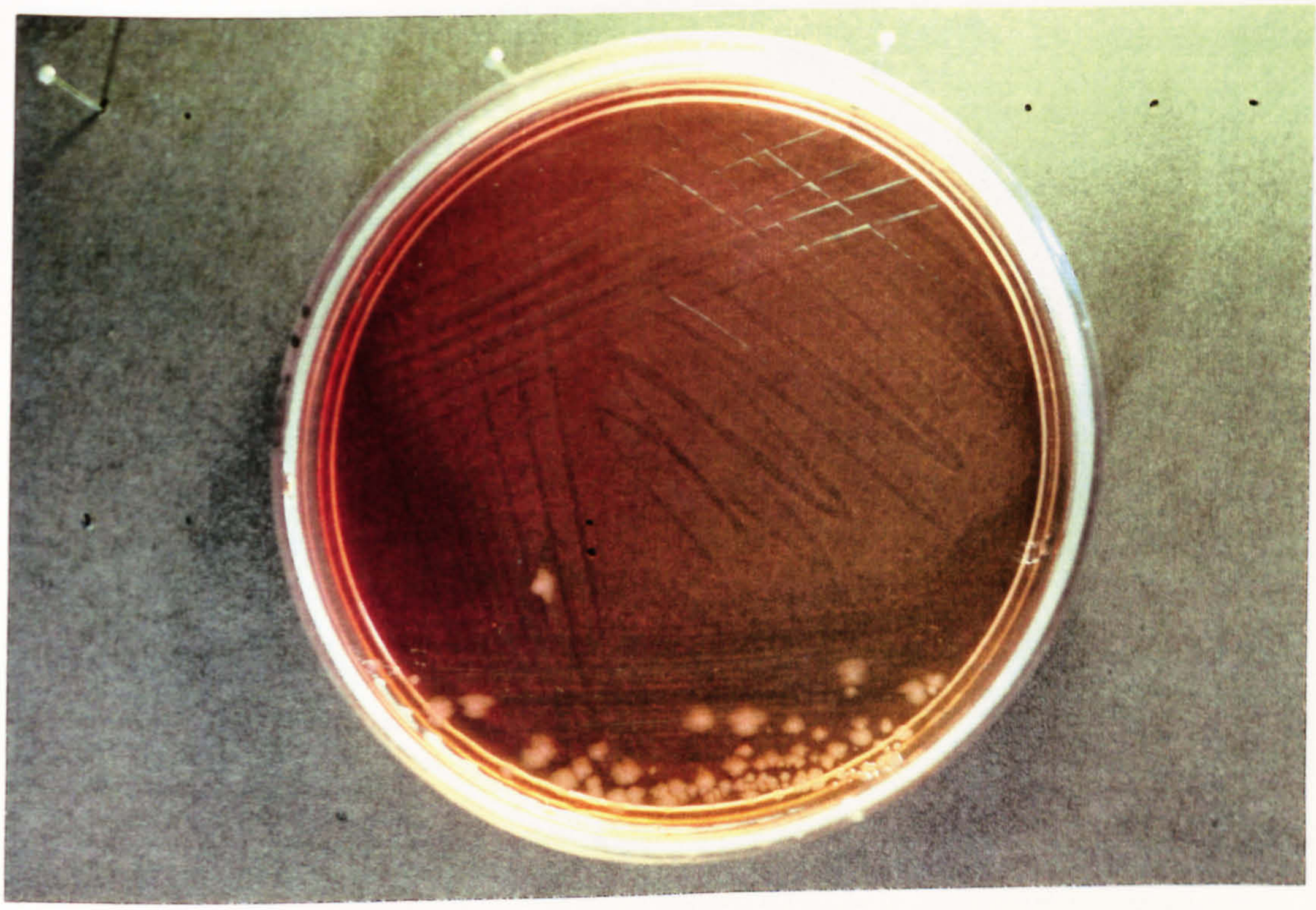


Plate 8.0 Comparison Of Appearence Of *Shig. sonnei* (426) On MC and XLD plates.



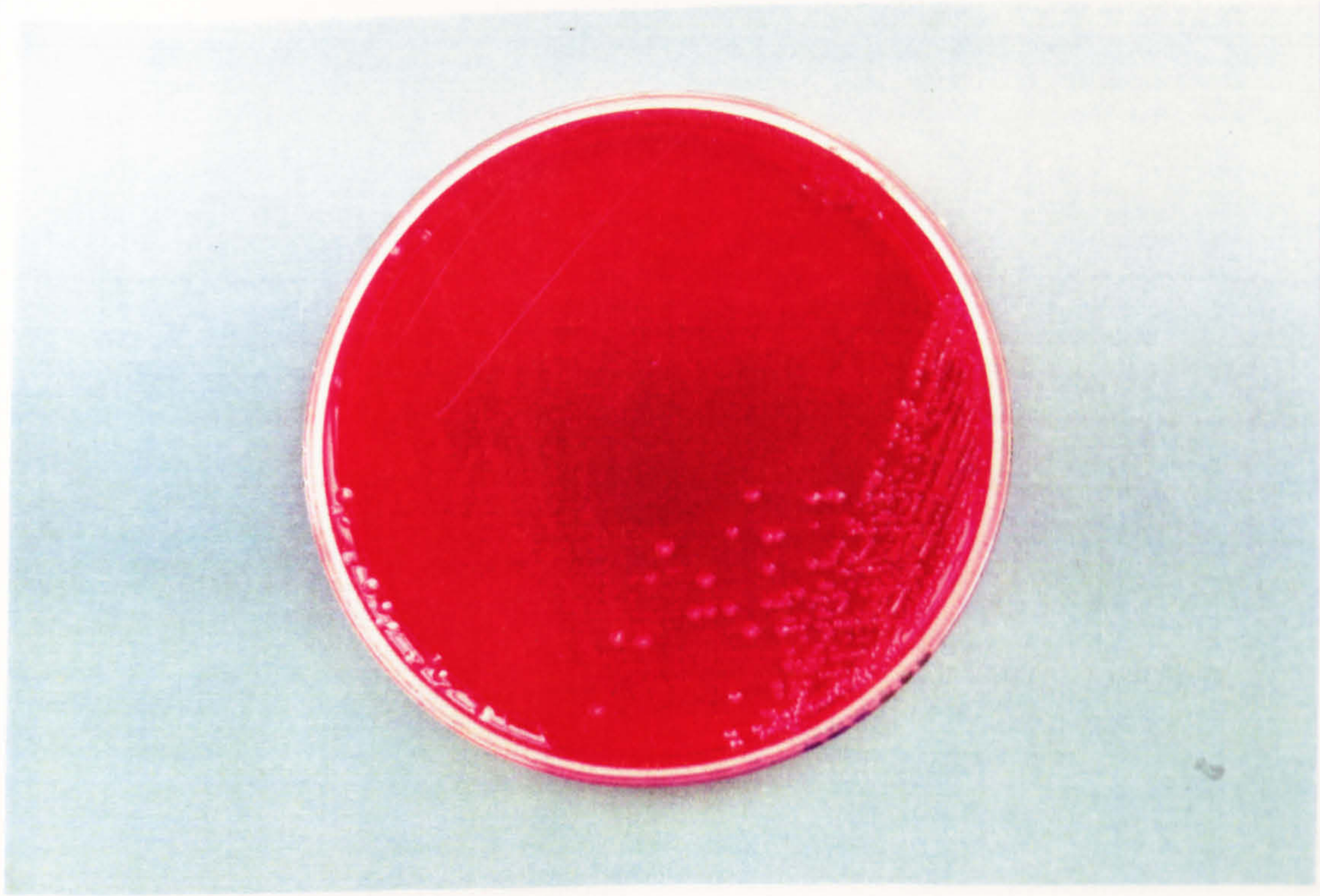
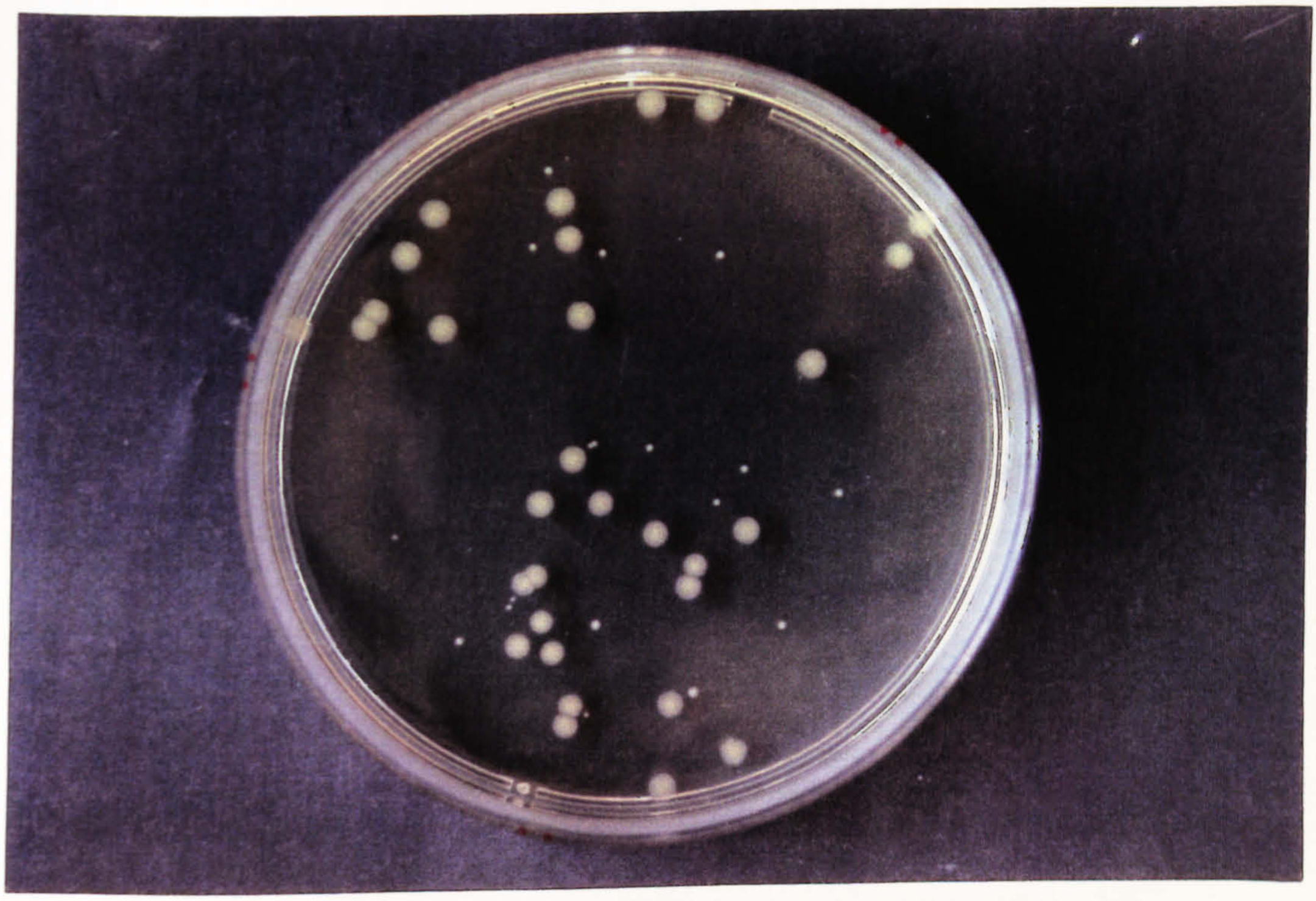


Plate 9.0 Comparison Of Isolated Bacteria from Ileostomy Effluents On TSA plates.



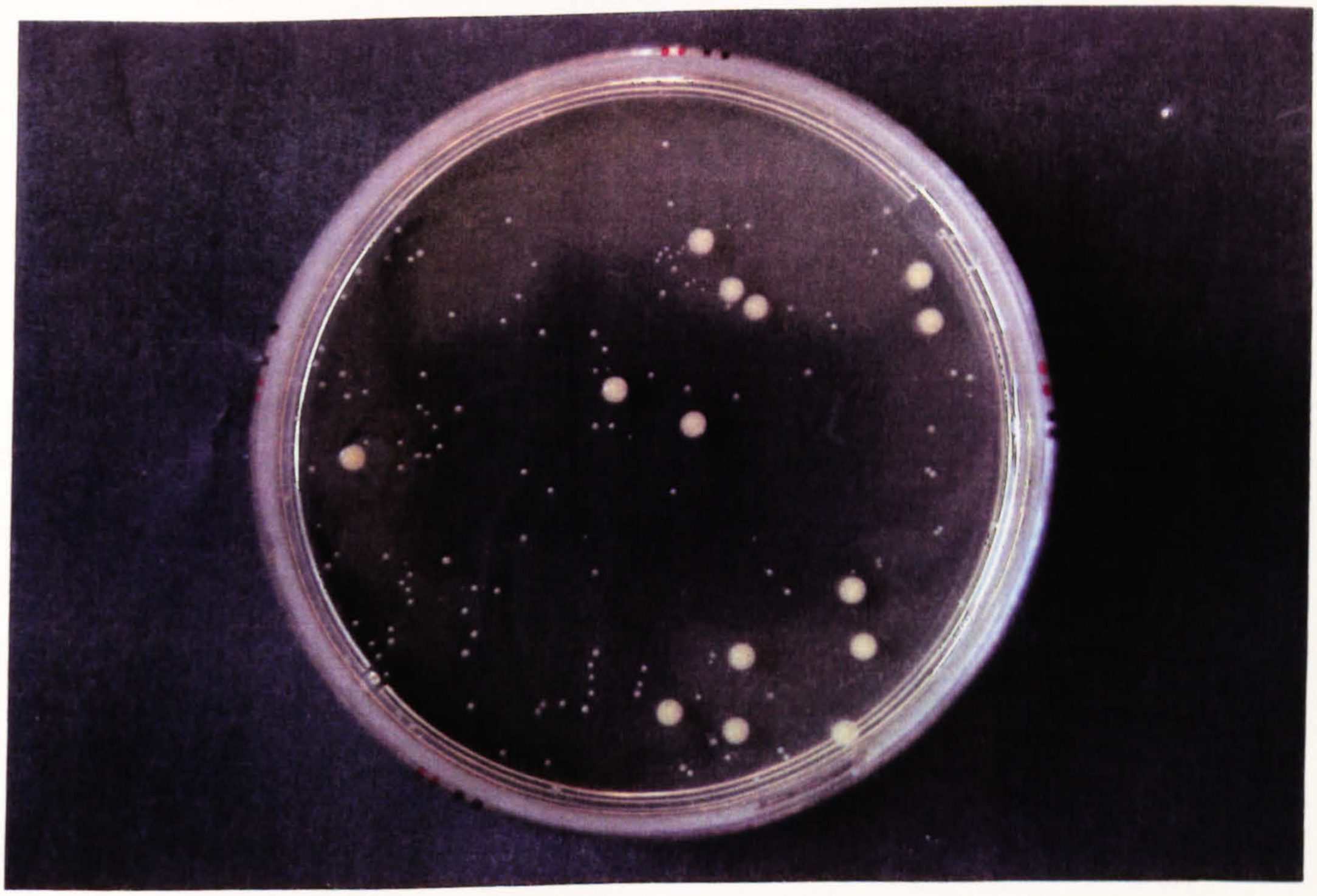


Plate 10.0 Comparison Of Isolated Bacteria from Ileostomy Effluents On BA plates.

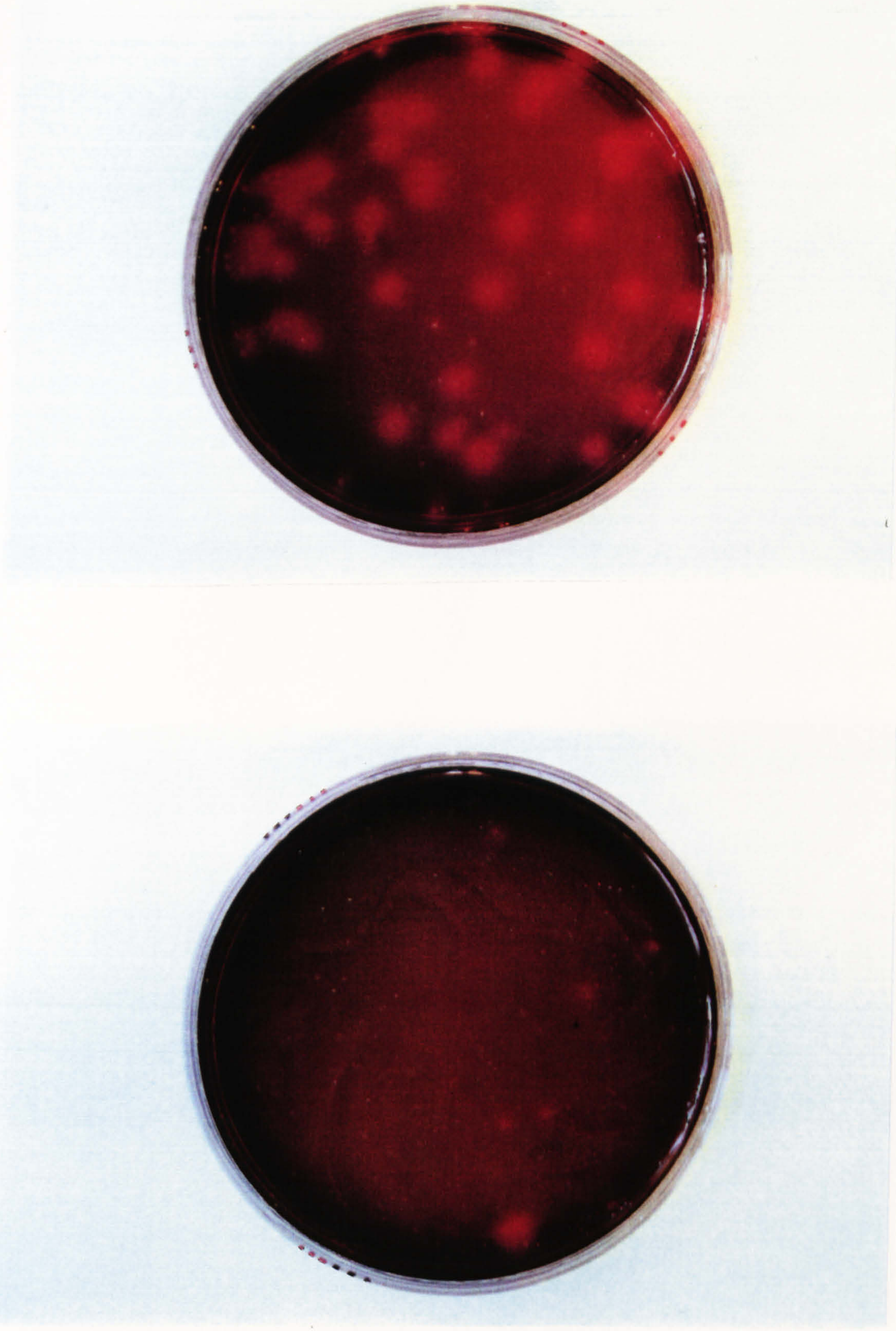
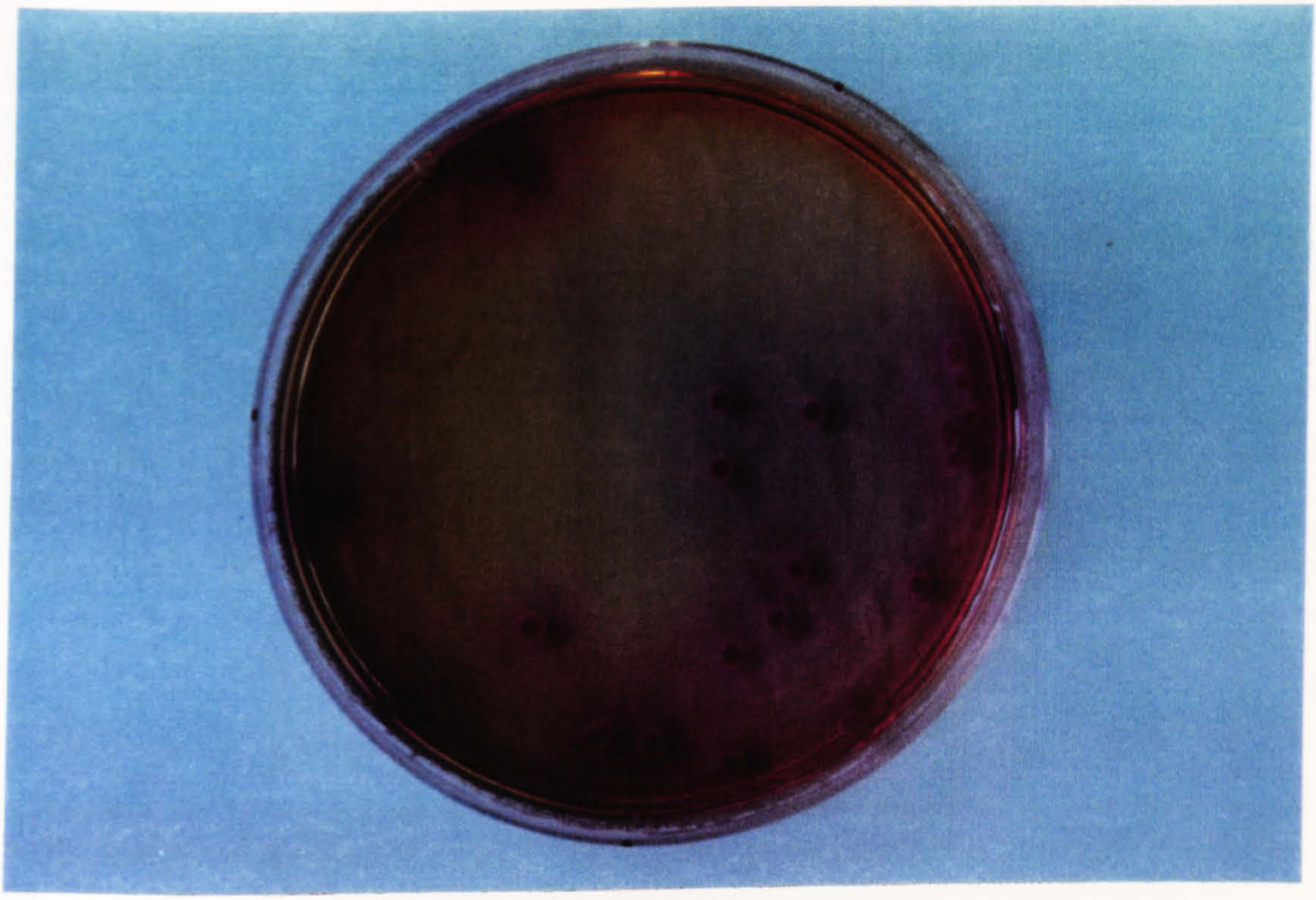


Plate 11.0 Comparison Of Isolated Bacteria from Ileostomy Effluents On MC plates.



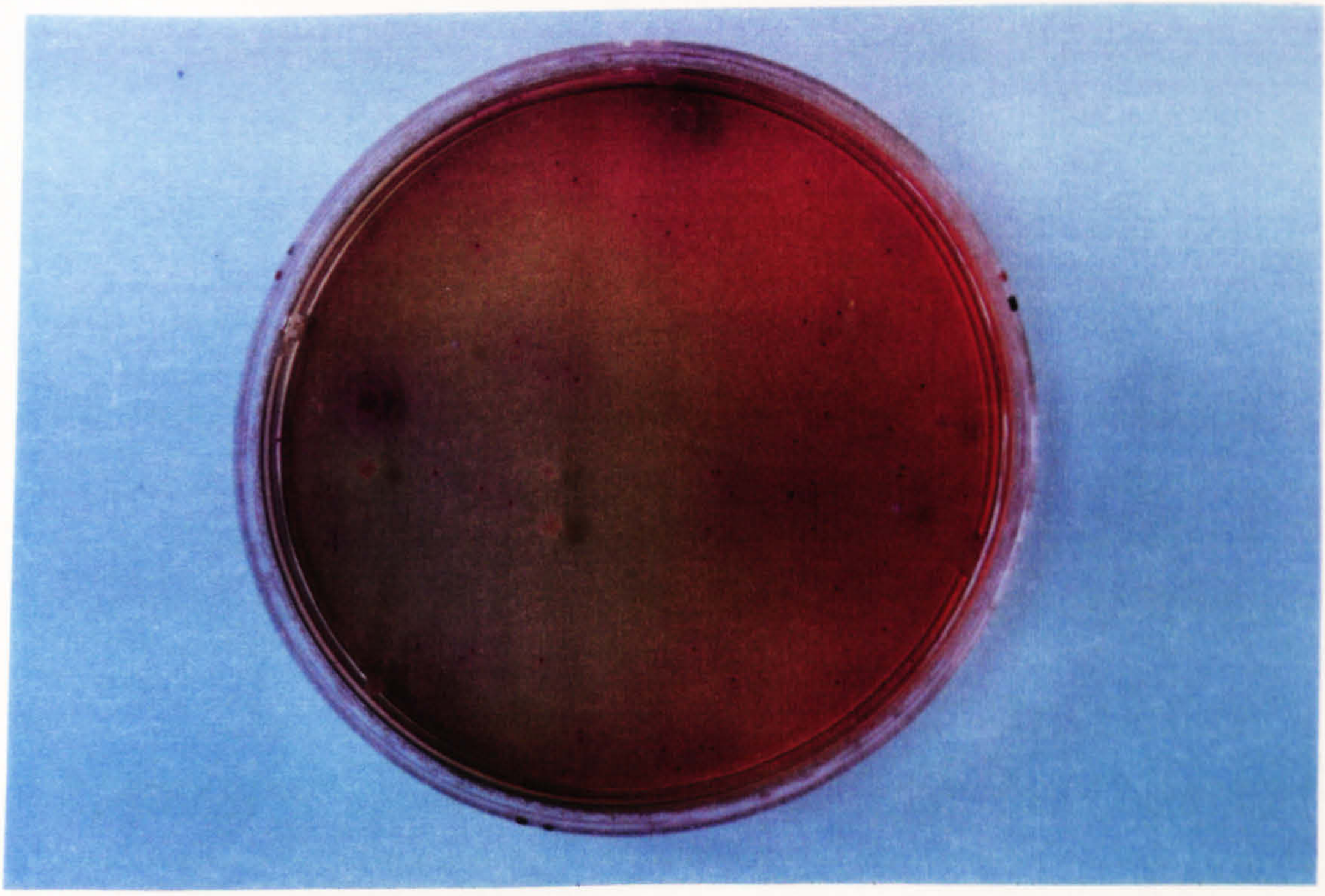
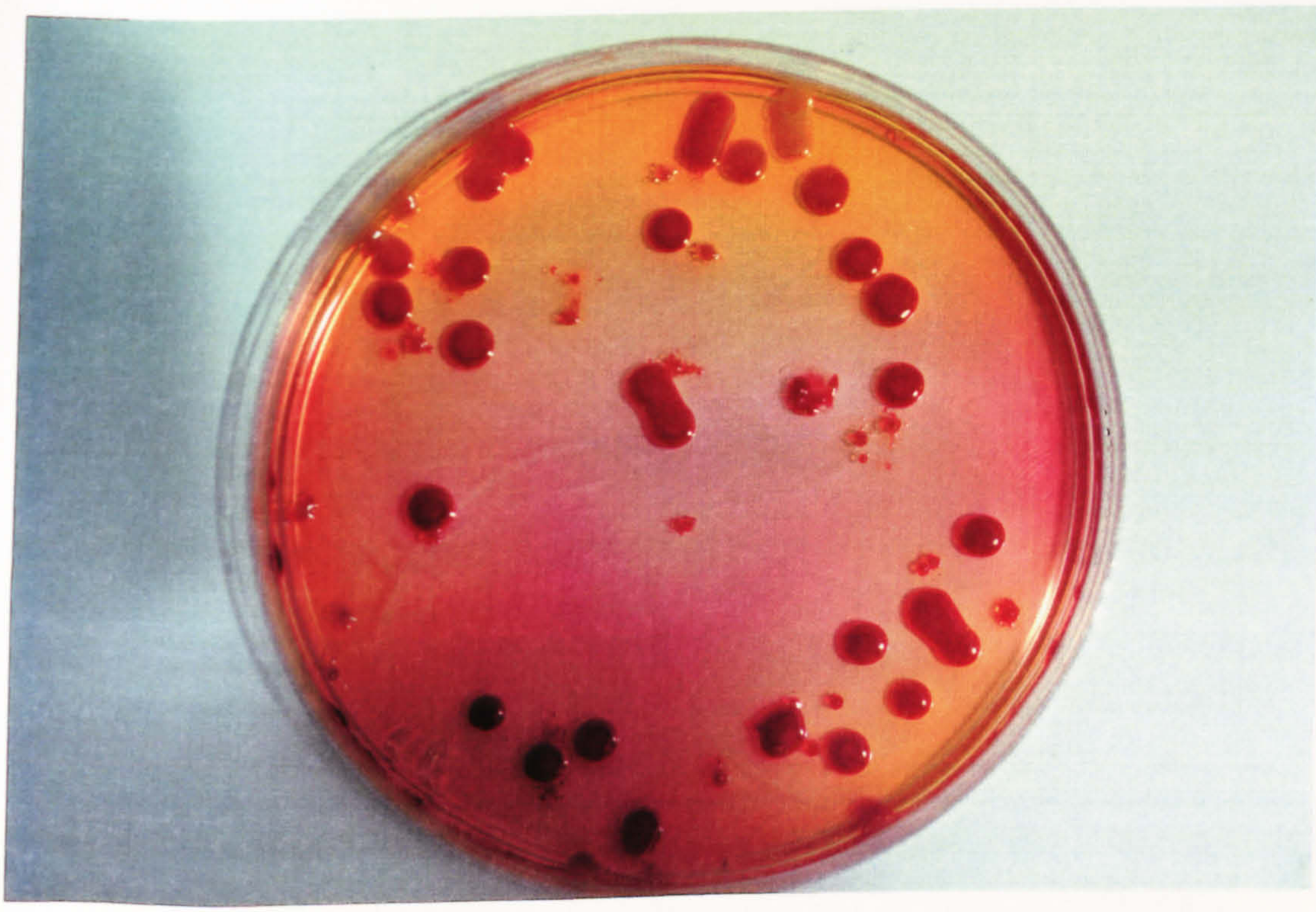


Plate 12.0 Closer Inspection Of Isolated Bacteria from Ileostomy Effluents On MC plates.



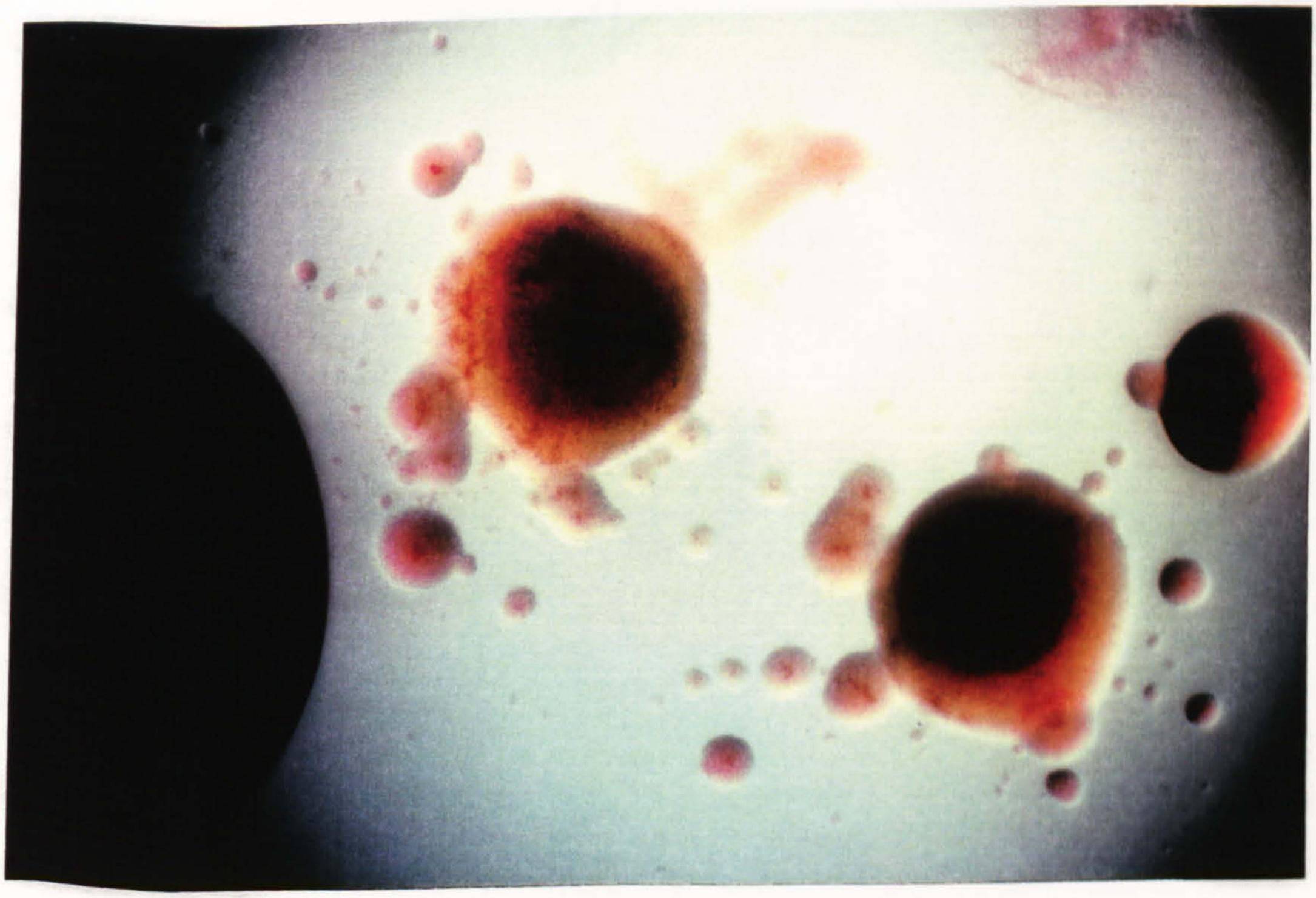


Plate 13.0 Closer Inspection Of Isolated Bacteria from Ileostomy Effluents On BA plates.

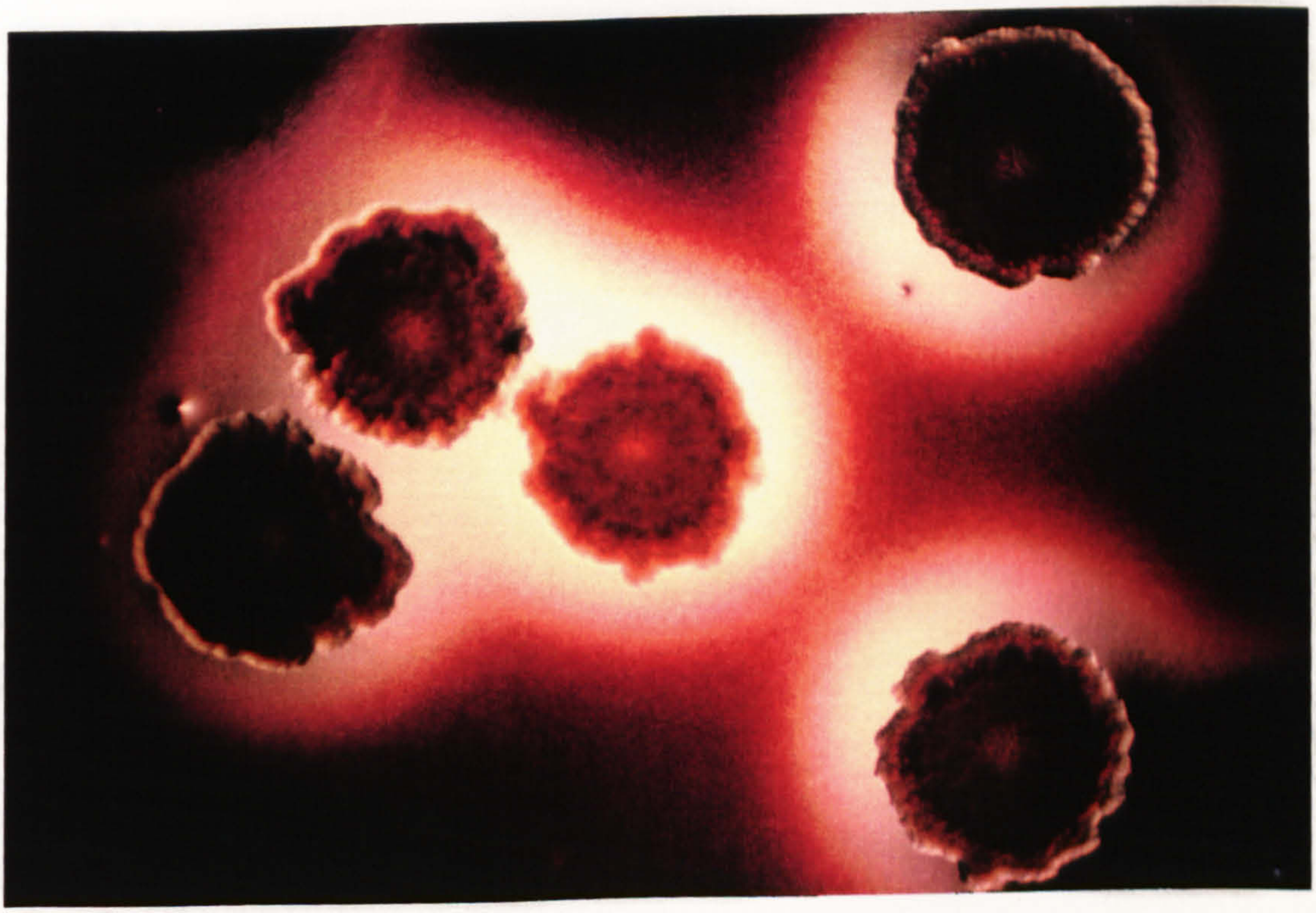
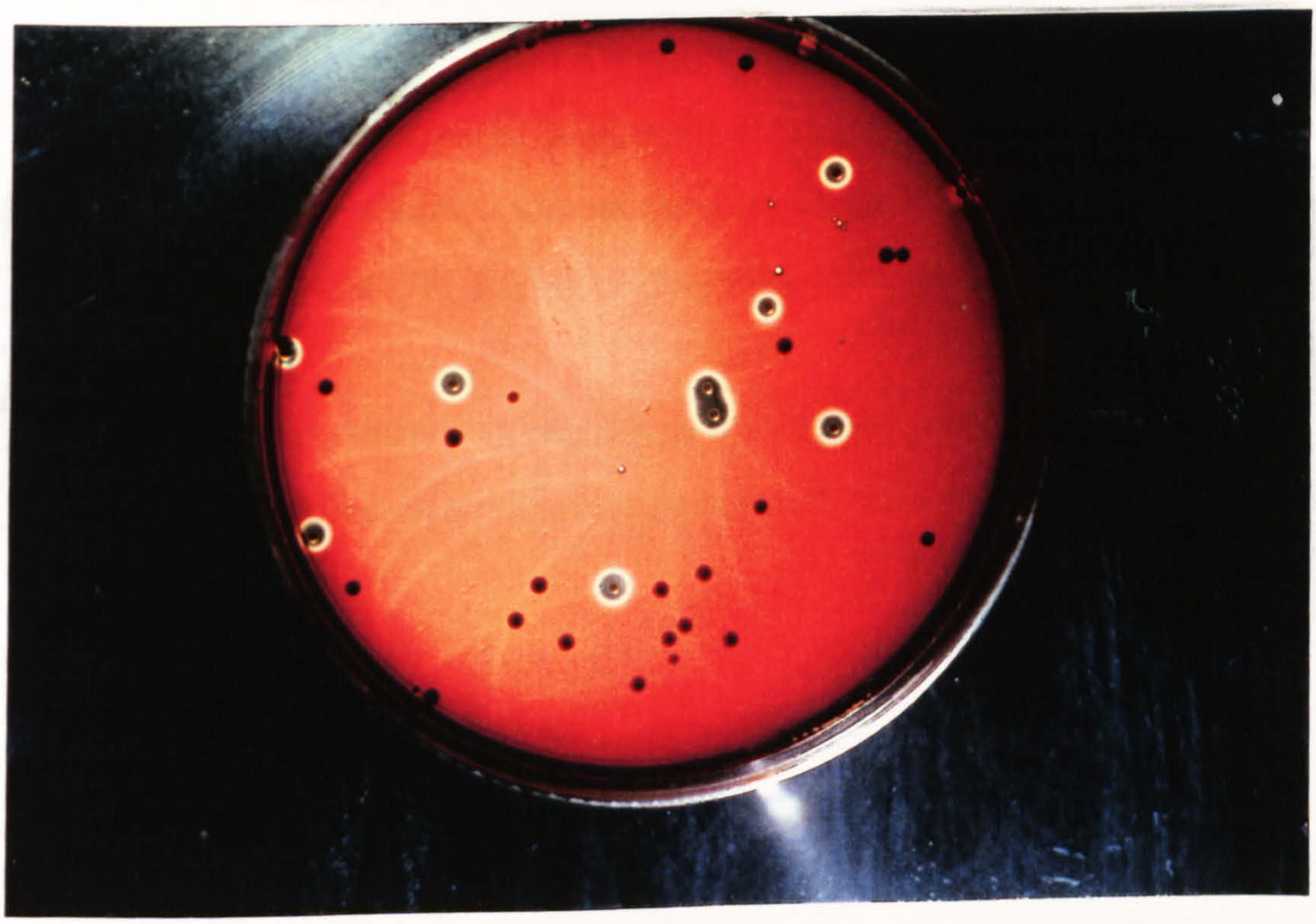
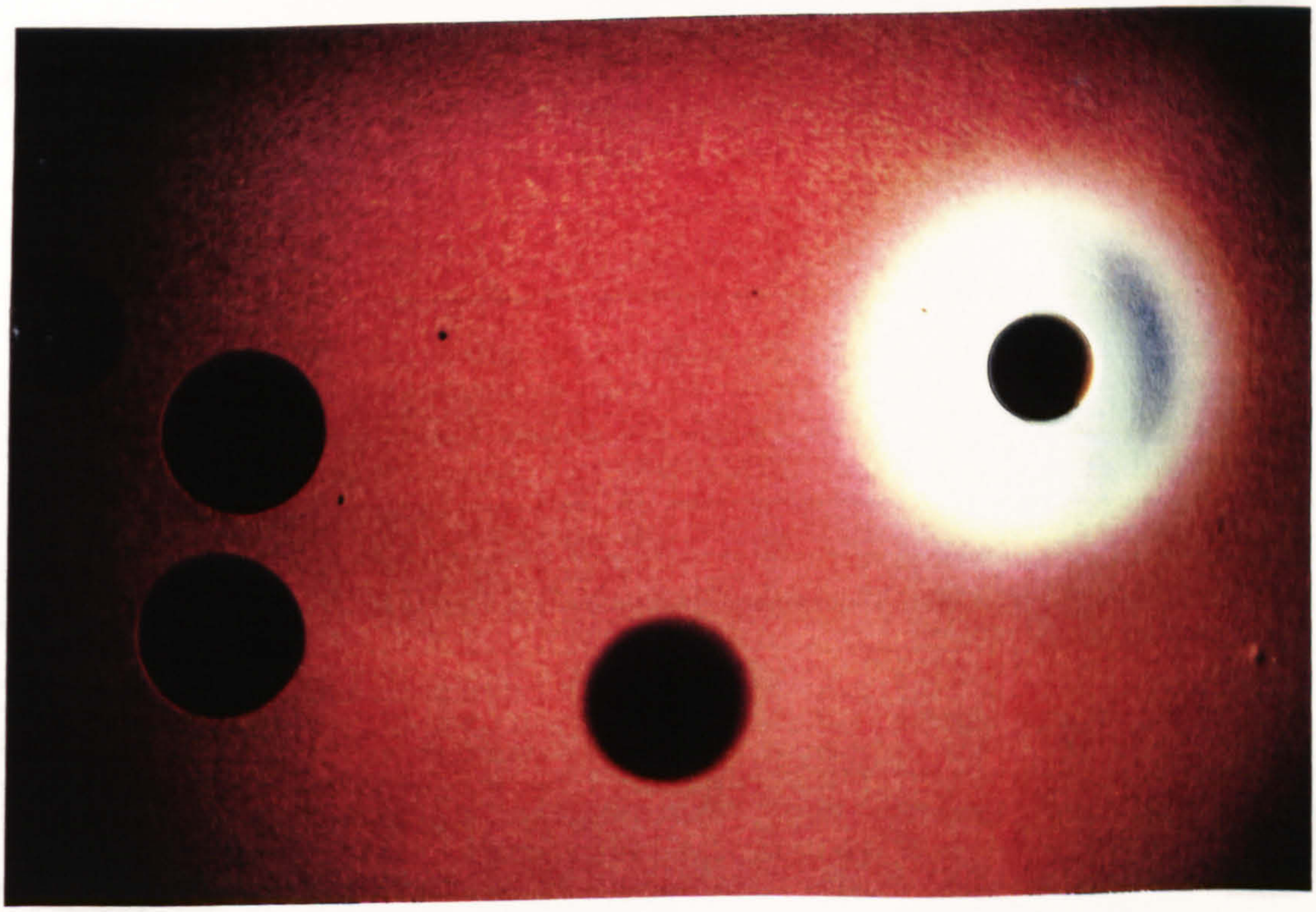


Plate 14.0 Closer Inspection Of Isolated Bacteria from Ileostomy Effluents On B A plates.





APPENDIX 4

Calculation of total sulphur compounds in G.P

Sulphide contents were calculated using dipropyl disulphide as internal standard and relative GC FID response factor was incorporated into the calculations. Alliin and Allicin contents were calculated from sulphide content based sulphur balance.

$$\text{Allicin content} = \Sigma[(\text{sulphide content} \times 162 \times N_s)/(M_s \times 2)]$$

$$\text{Alliin content} = \Sigma[(\text{allicin content} \times 177 \times 2)/(162)]$$

where:

162 = molecular weight of allicin,

177 = molecular weight of alliin,

M_s = molecular weight sulphide,

N_s = number of sulphur atoms in the sulphide.

APPENDIX 5

Sulphide Contents Of G.O

Calculation of the weight content from the RRF (Relative FID Response Factor).

The RRF of garlic sulphides to the internal standard was determined at Humberside University and are shown below.

Sulphide	Molecular Weight	Carbon No'	RRF
Dipropyl disulphide (IS)	150	6	1.000
Dimethyl disulphide	92	2	1.840
Diallyl sulphide	114	6	0.760
Methyl allyl disulphide	120	4	1.200
Dimethyl trisulphide	126	2	2.520
Diallyl disulphide	146	6	0.973
Methylallyl trisulphide	152	4	1.520
Diallyl trisulphide	178	6	1.187

The weight content was calculated as follows:

$$\text{corrected weight content (mg/ml G.O)} = \frac{A_{\text{sample sulphide}} \times W_{\text{is}}}{A_{\text{is}} \times W_{\text{sample}}} \times 1000 \times \text{RRF}$$

where $A_{\text{sample sulphide}}$ = peak area of sample sulphide

A_{is} = peak area of internal standard

W_{sample} = sample weight (ml)

W_{is} = weight of internal standard (mg)

CHAPTER 7

BIBLIOGRAPHY

Abdou, I.A., Abou-Zeid, A.A., El-Sherbeeny, M.R. & Abou-El-Gheat, Z.H. (1972)
Antimicrobial activities of *Allium sativum*, *Allium cepa*, *Rhapanus sativus*, *Capsicum frutescens*, *Eruca sativa* and *Allium kurrat* on bacteria.
Qualitative Plant Materials and Vegetation 22 pp29-35.

Adetumbi, M., Javor, G.T. & Lau, B.H.S. (1986)
Allium sativum (Garlic) inhibits lipid synthesis by *Candida albicans.*
Antimicrobial Agents And Chemotherapy 30 (3) pp499-501.

Agrawal, P. (1978)
Effect of root and bulb extracts of *Allium spp.* on fungal growth.
Trans British Mycology Society 70 pp439-442.

Aly, R. & Shinefield, H.R. (1982)
Bacterial Interference
CRC Press. Boca Ration, Florida

Amonkar, S.V. & Reeves, E.L. (1970)
Mosquito control with active principle of garlic, *Allium sativum.*
Journal of Economical Entomology 63 pp1172-1175

Amonkar, S.V. & Banerji, A. (1971)
Isolation and characterization of lavicidal principle of garlic.
Science 174 pp1343-1344.

Apella, M.C., González, S.N., Nader de Marcías, M.E., Romero, N. & Oliver, G. (1992)
In vitro studies on the inhibition of the growth of *Shigella sonnei* by *Lactobacillus casei* and *Lactobacillus acidophilus.*
Journal of Applied Bacteriology 73 pp480-483.

Appleton. J.A. & Tansey, M.R. (1975)
Inhibition of growth of zoopathogenic fungi by garlic extract.
Mycologia 67 pp882-885.

Aries, V., Crowther, J.S., Drasar, B.S. & Hill, M.J. (1969)
Degradation of bile salts by human intestinal bacteria.
Gut 10 pp575-576.

Atlas, R.M. (1989)
Microbiology: Fundamentals and Applications
Second Edition Maxwell Macmillian International Editions. New York.

Baird-Parker, A.C. (1990)
Foodborne illness - foodborne salmonellosis.
The Lancet 336 Nov17th pp1231-1235.

Banwell, J.G., Gorbach, S.L., Pierce, N.F., Mitra, R. & Mondal, A. (1971)
Acute undifferentiated human diarrhea in the tropics. II. alterations in intestinal fluid and electrolyte movements.
The Journal of Clinical Investigation 50 pp890-900.

Barefoot, S.F. & Klanenhammer, T.R. (1983)
Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*.
Applied & Environmental Microbiology 45 pp1808-1815.

Barlow, P.J., Yan, X. & Wang, Z. (1993)
Garlic preparations and their analysis.
Garlic In Focus - A Press Conference.

Barone, F.E. & Tansey, M.R. (1977)
Isolation, purification, identification, synthesis and kinetics of activity of the anticandidal component of *Allium sativum*, and a hypothesis for its mode of action.
Mycologia 69 pp793-825.

Beachey, E.H. (1981)
Bacterial adherence: adhesin-receptor interactions mediate the attachment of bacteria to mucosal surfaces.
Journal of Infectious Diseases 143 (3) pp325-345.

Bean, H.S. & Das, A. (1966)
The adsorption by *Escherichia coli* of phenols and their bactericidal activity.
Journal of Pharmacy and Pharmacology 11 pp195T-197T.

Belman, S. (1983)
Onion and garlic inhibit tumor promotion.
Carcinogenesis 4 pp1063-1065.

Beuchat, L.R. (1976)
Sensitivity of *Vibrio parahaemolyticus* to spices and organic acids.
Journal of Food Science 41 pp899-902.

Binder, H.J., Filburn, B. & Floch, M.H. (1975)
Bile acids inhibition of intestinal anaerobic organisms.
American Journal of Clinical Nutrition 28 pp119-125.

Block, E. (1985)
The chemistry of garlic and onions.
Scientific American 252 (3) pp94-99.

Block, E., Iyer, R., Grisoni, S., Saha, S., Belman, S. & Lossing, F.P. (1988)
Lipoxygenase inhibitors from the essential oil of garlic. Markovnikov addition of the allyl dithio radical to olefins.
Journal of the American Chemical Society 110 pp7813-7827.

Bogin, E., Abrams, M. & Earon, Y. (1984)
Effect of garlic extract on red blood cells.
Journal of Food Protection 47 (2) pp100-101,104.

Briozzo, J., Núñez, L., Chirife, J., Herszage, L. & Aquino, M.D. (1989)
Antimicrobial activity of clove oil dispersed in a concentrated sugar solution.
Journal of Applied Bacteriology 66 pp69-75.

Brock, T.D. (1966)
Principles of Microbial Ecology
Prentice-Hall, Englewood Cliffs. New Jersey.

Brockett, M. & Tannock, G.W. (1982)
Dietary influence on microbial activites in the caecum of mice.
Canadian Journal of Microbiology 28 pp493-499.

Brodnitz, M.M., Pascale, J.V. & Van Derslice, L. (1971)
Flavor components of garlic extract.
Journal of Agricultural Food Chemistry 19 (2) pp273-275.

Brown, W.R., Savage, D.C., Dubois, R.S., Alp, M.H., Mallory, A. & Kern, F. (1972)
Intestinal microflora of immunoglobulin-deficient and normal human subjects.
Gastroenterology 62 (6) pp1143-1152.

Bullen, C.L., Tearle, P.V. & Willis, A.T. (1976)
Bifidobacteria in the intestinal tract of infants: an *in vivo* study.
Journal of Medical Microbiology 9 pp325-333.

Bullen, C.L., Tearle, P.V. & Stewart, M.G. (1977)
The effect of "humanised" milks and supplemented breast feeding on the faecal flora of infants.
Journal of Medical Microbiology 10 (4) pp403-413.

Burrows, W. (1968)
Cholera toxins.
Annual Review of Microbiology 22 pp245-268.

Caporaso, N., Smith, S.M. & Eng, R.H.K. (1983)
Antifungal activity in human urine and serum after ingestion of garlic (*Allium sativum*).
Antimicrobial Agents and Chemotherapy 23 (5) pp700-702.

Cash, R.A., Music, S.I., Libonati, J.P., Synder, M.J., Wenzel, R.P. & Hornick, R.B. (1974)
Response of man to infection with *Vibrio cholerae*. I. Clinical, serological and bacteriological responses to a known inoculum.
Journal of Infectious Diseases 129 pp45-52

Cavallito, C.J. (1946)
Relationship of thiol structures to reaction with antibiotics.
Journal of Biological Chemistry 164 pp29-34.

Cavallito, C.J. & Bailey, J.H. (1944)
Allicin, the antibacterial principle of *Allium sativum*.
I. Isolation, physical properties and antibacterial action.
Journal of the American Chemical Society 66 pp1950-1951.

Cavallito, C.J., Buck, J.S. & Suter, C.M. (1944)
Allicin, the antibacterial principle of *Allium sativum*.
II. Determination of the chemical structure.
Journal of the American Chemical Society 66 pp1952-1954.

Cavallito, C.J., Bailey, J.H. & Buck, J.S. (1945)
Allicin, the antibacterial principle of *Allium sativum*.
III Its precursor and "essential oil" of garlic.
Journal of the American Chemical Society 67 pp1032-1033.

Chaney, A.L. & Marbach, E.P. (1962)
Modified reagents for determination of urea and ammonia.
Clinical Chemistry 8 (2) pp130-132.

Cheng, K.J., Irvin, R.T & Costerton, J.W. (1981)
Autochthonous and pathogenic colonization of animal tissues by bacteria.
Canadian Journal of Microbiology 27 pp461-490.

Chowdhury, A.K.A., Ahsan, M., Islam, N.S. & Ahmed, Z.U. (1991)
Efficiacy of aqueous extract of garlic and allicin in experimental shigellosis in rabbits.
Indian Journal of Medical Research [A] 93 pp33-36.

Clarke, R.T.J. (1977)
Methods for studying gut microbes. IN Microbial Ecology of the Gut
Clarke, R.T.J. & Bauchop, T. (Eds) Academic Press, London & New York.

Coleman, R. (1987)
Bile salts and bilary lipids.
Biochemical Society Transactions 15 pp68S-80S.

Communicable Disease Surveillance Centre Report (1991)
E. coli 0157
Communicable Disease Report 1 35.

Copperstock, M.S. & Zedd, A.J. (1983)
Intestinal flora of infants. IN Human Intestinal Microflora in Health and Disease
Hentges, D.J. (Ed) Academic Press. New York. pp79-93.

Cota-Robels, E.H. (1963)
Electron microscopy of plasmolysis in *Escherichia coli.*
Journal of Bacteriology 85 pp499-503.

Criss, W.E., Fakunle, J., Knight, E., Adkins, J., Morris, H.P. & Dhillon, G. (1982)
Inhibition of tumor growth with low dietary protein and with dietary garlic extracts.
Proceedings of the 66th Annual Meeting of American Society of Experimental Biology 41 (Abst 74) pp281.

Croshaw, B. (1977)
Evaluation of non-antibiotic antimicrobial agents. IN Pharmaceutical Microbiology
Hugo, W.B. & Russel, A.D. (Eds) Blackwell Scientific Publications. Oxford. pp185-201.

Csonka, L.N. (1989)
Physiological and genetic responses of bacteria to osmotic stress.
Microbiological Reviews 53 (1) pp121-147.

Cummings, J.H. & Macfarlane, G.T. (1991)
The control and consequences of bacterial fermentation in the human colon.
Journal of Applied Bacteriology 70 pp443-459.

Dabaneh, B.F.A & Al-Delaimy, K.S. (1984)
Inhibition of *Staphylococcus aureus* by garlic extract.
Lebensm. wiss. U. Technol 17 pp29-31.

Dankert, J., Tromp, T.F.J., Vries, H. De. & Klasen, H.J. (1979)
Antimicrobial activity of crude juices of *Allium ascalonicum*, *Allium cepa* and *Allium sativum*.
Zentralblatt fur Bakter Parasitenkunde 1a pp229-239.

Davenport, H.W. (1985)
Physiology of the digestive tract.
Chicago. Year Book Medical Publishers.

Delaha, E.C. & Garagusi, V.F. (1985)
Inhibition of Mycobacteria by garlic extract (*Allium sativum*).
Antimicrobial Agents and Chemotherapy 27 (4) pp485-486.

De Wit, J.C., Notermans, S., Gorin, N. & Kampelmacher, E.H. (1979)
Effect of garlic oil or onion oil on toxin production in meat slurry.
Journal of Food Protection 42 (3) pp222-224.

Dixon, J.M.S. (1960)
The fate of bacteria in the small intestine.
Journal Pathology and Bacteriology 79 pp131-140.

Dorant, E., van den Brant, P.A., Goldbohm, R.A., Hermus, R.J.J. & Sturmans, F. (1993)
Garlic and its significance for the prevention of cancer in humans: a critical review.
British Journal of Cancer 67 pp424-429.

Doty, R.L. (1981)
Olfactory communication in humans.
Chemical Senses 6 pp351-376.

Drasar, B.S. (1974)
The Normal Microbial Flora of Man.
Skinner, F.A. & Carr, J.G. (Eds) Academinc Press. London. pp187-196.

Drasar, B.S. & Crowther, I.S (1971)
The cultivation of human intestinal bacteria. IN Isolation of Anaerobes
Shapton, D.A & Boards, B.G. (Eds) Academic Press. New York. pp93-97.

Drasar, B.S. & Barrow, P.A. (1985)
Intestinal Microbiology: Aspects of Microbiology 10.
Van Nostrand Reinhold. UK Co.Ltd.

Drasar, B.S. & Hill, M.J. (1974)
Human Intestinal Flora. Academic Press. London.

Drasar, B.S., Shiner, M. & McLeod, G.M. (1969)
Studies on the intestinal flora I. The bacterial flora of the gastrointestinal tract in healthy and achlorhydric persons.
Gastroenterology 56 (1) pp71-79.

Dubos, R., Schaedler, R.W., Costello, R. & Hoet, P. (1965)
Indigenous, normal and autochthonous flora of gastrointestinal tract.
Journal of Experimental Medicine 122 pp67-77.

Ducluzeau, R., Dubos, F., Raibaud, P. & Abrams, G.D. (1976)
Inhibition of *Clostridium perfringens* by an antibiotic substance produced by *Bacillus licheniformis* in the digestive tract of gnobiotic mice: effect on other bacteria from the digestive tract.
Antimicrobial Agents and Chemotherapy 9 (1) pp20-25.

Duerdon, B.I (1980)
The isolation and identification of *Bacteroides spp.* from the normal human faecal flora.
Journal of Medical Microbiology 13 pp69-78.

Egen-Schwind, C., Eckland, R. & Kemper, F.H. (1991)
Metabolism of allicin in the isolated perfused rat liver.
Second International Garlic Symposium: Pharmacy, Pharmacology and Clinical Application of *Allium sativum.* Berlin 7-10 March. pp13.

Elnima, E.I., Ahmed, S.A., Mekkawi, A.G. & Mossa, J.S. (1983)
The antimicrobial activity of garlic and onion extracts.
Pharmazie 38 (11) pp747-748.

Feldberg, R.S., Chang, S.C., Kotik, A.N., Nadler, M., Neuwirth, Z., Sundstrom, D.C. & Thompson, N.H. (1988)
In vitro mechanism of inhibition of bacterial cell growth by allicin.
Antimicrobial Agents and Chemotherapy 32 (12) pp1763-1769.

Fenwich, G.R. & Hanley, A.B. (1985a)
The Genus *Allium* - Part 1.
CRC Critical Reviews in Food Science and Nutrition 22 (3) pp199-271.

Fenwick, G.R. & Hanley, A.B. (1985b)
The Genus *Allium* - Part 2.
CRC Critical Reviews in Food Science and Nutrition 22 (4) pp273-377.

Fenwick, G.R. & Hanley, A.B. (1985c)
The Genus *Allium* - Part 3.
CRC Critical Reviews in Food Science and Nutrition 23 (1) pp1-73.

Field, M. (1979)
Modes of action of enterotoxins from *Vibrio cholerae* and *Escherichia coli*.
Review of Infectious Diseases 1 pp918-926.

Finegold, S.M. (1969)
Intestinal bacteria. The role they play in normal physiology, pathogenic physiology and infection.
California Medica 110 pp455-459.

Finegold, S.M., Sutter, V.L., Boyle, J.D. & Shimada, V.L. (1970)
The normal flora of ileostomy and transverse colostomy effluents.
The Journal of Infectious Diseases 122 (5) pp376-381.

Finegold, S.M., Sutter, V.L. & Mathisen, G.E. (1983)
Normal indigenous intestinal flora. IN Human Intestinal Microflora in Health and Disease
Hentges, D.J. (Ed) Academic Press. London. pp3-31.

Floch, M.H., Gershengoren, W., Elliot, S. & Spiro, H.M. (1971)
Bile acid inhibition of the intestinal microflora - A function for simple bile acids.
Gastroenterology 61 (2) pp228-233.

Floch, M.H., Binder, H.J., Filburn, B. & Gershengoren, W. (1972)
The effect of bile acids on intestinal microflora.
American Journal of Clinical Nutrition 25 pp1418-1426.

Focke, M., Feld, A. & Lichtenthaler, H.K. (1990)
Allicin, a naturally occuring antibiotic from garlic, specifically inhibits acetyl-CoA synthetase.
Federation of European Biochemical Societies 261 (1) pp106-108.

Freter, R. (1975a)
Interactions between mechanisms controlling the intestinal microflora.
American Journal of Clinical Nutrition 27 pp1409-1416.

Freter, R. (1975b)
Microbiology
Schlessinger, D. (Ed) American Society of Microbiology, Washington D.C pp154-157.

Freter, R. (1983)
Mechanisms that control the microflora in the large intestine. IN Human Intestinal Flora in Health and Disease
Hentges, D.J. (Ed) Academic Press. London. pp33-54.

Freter, R. & Abrams, G.D. (1972)
Functions of various intestinal bacteria in converting germ-free mice to the normal state.
Infection and Immunity 6 pp119-126.

Freter, R., Brickner, H., Botney, M., Cleven, D. & Aranki, A. (1983)
Mechanisms that control bacterial populations in continuous flow culture models of mouse large intestine flora.
Infection and Immunity 39 (2) pp676-685.

Fulder, S. (1990)
Garlic as a medicine.
A Supplement To Chemist & Druggist March17th.

George, K.C., Amonkar, S.V. & Eapen, J. (1973)
Effect of garlic oil on incorporation of amino acids into proteins of *Cupex bipiens quinquefasciatus Say.* larvae.
Chemical-Biological Interactions 6 pp169-175.

George, K.C. & Eapen, J. (1974)
Mode of action of garlic oil. Effect on oxidative phosphorylation in hepatic mitochondria in mice.
Biochemical Pharmacology 23 pp931-936.

Ghannoum, M.A. (1988)
Studies on the anticandidal mode of action of *Allium sativum* (garlic).
Journal of General Microbiology 134 pp2917-2924.

Ghannoum, M.A. (1990)
Inhibition of *Candida* adhesion to buccal epithelial cells by an aqueous extract of *Allium sativum* (garlic).
Journal of Applied Bacteriology 68 pp163-169.

Giannella, R.A., Broitmann, S.A. & Zamcheck, N. (1972)
Gastric acid barrier to ingested microorganisms in man: Studies *in vivo* and *in vitro*.
Gut 13 pp251-256.

Gibson, G.R., Macfarlane, G.T. & Cummings, J.H. (1988a)
Occurance of sulphate-reducing bacteria in human faeces and the relationship of dissimilatory sulphate reduction to methanogenesis in the large gut.
Journal of Applied Bacteriology 65 pp103-111.

Gibson, G.R., Cummings, J.H. & Macfarlane, G.T. (1988b)
Competition for hydrogen between sulfate-reducing bacteria and methanogenic bacteria from the human large intestine.
Journal of Applied Bacteriology 65 pp241-247.

Gorbach, S.L. (1971)
Progress in gastroenterology: intestinal microflora.
Gastroenterology 60 (6) pp1110-1129.

Gorbach, S.L. & Etkin, S. (1970)
Studies on enterotoxin from *Escherichia coli* associated with acute diarrhea in man.
Journal of Laboratory Clinical Medicine 76 (6) pp999-1000.

Gorbach, S.L., Nahas, L., Lerner, P.I. & Weinstein, L. (1967a)
Studies of intestinal microflora I. Effects of diet, age and periodic sampling on numbers of faecal microorganisms in man.
Gastroenterology 53 (6) pp845-855.

Gorbach, S.L., Plaut, A.G., Nahas, L., Weinstein, L., Spanknel, G. & Levitan, R. (1967b)
Studies of intestinal microflora II. Microorganisms of the small intestine and their relations to oral and fecal flora.
Gastroenterology 53 (6) pp856-867.

Gorbach, S.L., Nahas, L., Weinstein, L., Levitan, R. & Patterson, J.F. (1967c)
Studies of intestinal microflora IV. The microflora of ileostomy effluent: a unique microbial ecology.
Gastroenterology 53 (6) pp874-880.

Gorbach, S.L. & Tabaqchali, S. (1969)
Bacteria, bile and the small bowel.
Gut 10 pp963-972.

Gracey, M., Stone, D.E., Suharjono, & Sunoto. (1974)
Isolation of *Candida* species from the gastrointestinal tract in malnourished children.
The American Journal of Clinical Nutrition 27 (4) pp345-349.

Gray, J.D.A. & Shiner, M. (1967)
Influence of gastric pH on gastric and jejunal flora.
Gut 8 pp574-581.

Haenal, H. (1970)
Human normal and abnormal gastrointestinal flora.
American Journal of Clinical Nutrition 23 (11) pp1433-1439.

Harris, L.J., Daeschel, M.A., Stiles, M.E. & Klaenhammer, T.R. (1989)
Antimicrobial activity of lactic acid bacteria against *Listeria monocytogenes.*
Journal of Food Protection 52 pp384-387.

Hazell, S.L., Lee, A., Brady, L. & Hennessy, W. (1986)
Campylobacter pyloridis and gastritis: association with intracellular spaces and adaptation to an environment of mucus as important factors in colonization of the gastric epithelium.
Journal of Infectious Diseases 153 (4) pp658-663.

Hentges, D.J. (1983)
Human Intestinal Microflora In Health & Disease
Academic Press, New York & London.

Hentges, D.J. & Freter, R. (1962)
In vivo and *in vitro* antagonism of intestinal bacteria against *Shigella flexneri* I. Correlation between various tests.
Journal of Infectious Diseases 110 pp30-37.

Hill, D.J. & Maslin, D.J. (1991)
A comparison of antimicrobial properties of alliflex and interprise garlic powder extracts.
Industrial Report to Seven Seas Ltd.

Holdeman, L.V. & Moore, W.E.C. (1972)
Roll-tube techniques for anaerobic bacteria.
American Journal of Clinical Nutrition 25 (12) pp1314-1317.

Hörheimer, L., Wagner, H., Seith, M. & Vejdelek, Z.J. (1968)
Zur Wertbestimmung von Knoblauchpräparaten.
Pharmazie 23 pp462.

Howden, C.W. & Hunt, R.N. (1987)
Relationship between gastric secretion and infection.
Gut 28 pp96-107.

Kanezawa, A., Nakagawa, S., Sumiyoshi, H., Masamoto, K., Harda, H., Nakagami, S., Date, S., Yokkata, A., Nishkawa, M. & Fuwa, T. (1984)
General toxicity testing of a garlic extraction preparation containing vitamins.
Oyo Yakuri 27 pp909-911. (English Summary).

Karaioannoglou, P.G. (1977)
Effect of garlic extract on growth of vegetative cells and on outgrowth of spores of *Bacillus cereus.*
Hellvitica Vet Medica 22 pp119-128.

Karaioannoglou, P.G., Mantis, A.J. & Panetsos, A.G. (1977)
The effect of garlic extract on lactic acid bacteria (*Lactobacillus plantarum*) in culture media.
Lebensmittel Wiss Technol 10 pp148-150.

Kazaryan, R.A. & Goryachenkova, E.V. (1979)
Alliinase: purification and characterization.
Biokhimika 43 pp1502-1508.

Kice, J.L. & Rogers, T.E. (1974)
Mechanism of the alkaline hydrolysis of aryl thiosufinates and thiosulfonates.
Journal of American Chemical Society 96 pp8009-8015.

Kleijnen, J., Knipschild, P. & Teriet, G. (1989)
Garlic, onions and cardiovascular risk factors. A review of the evidence from human experiments with emphasis on commercially avialable preparations.
British Journal of Clinical Pharmacology 28 pp535-544.

Klimek, J.W., Cavallito, C.J. & Bailey, J.H. (1948)
Induced resistance of *Staphylococcus aureus* to various antibiotics.
Journal of Bacteriology 55 pp139-145.

Koch, V.H. & Jager, W. (1989)
Knoblauch.
Deutsche Apotheker Zeitung 129 (6) pp273-276.

Kumar, A. & Sharma, V.D. (1982)
Inhibitory effect of garlic (*Allium sativum Linn.*) on enterotoxigenic *Escherichia coli.*
Indian Journal of Medical Research 76 pp66-70.

Kutachi, H.C. (1988)
The gastrointestinal system. IN Physiology 2nd Edition.
Berne, R.H. & Levy, H.N. (Eds) C.V. Mosby Company. Washington DC. pp649-742.

Kumar, A. & Gupta, R.S. (1984)
A note on the sensitivity of enterotoxigenic *Staphylococcus aureus* for garlic extract (*Allium sativum Linn.*).
Indian Vetenary Journal 61 pp718-719.

Lau, B.H.S. (1989)
Anticoagulant and lipid regulating effects of garlic (*Allium sativum*). IN Current Topics in Nutrition and Disease
Liss, A.R. (Ed) vol 22, New York. pp295-325.

Lawson, L.D. (1993)
Bioactive organosulfur compounds of garlic and garlic products, and their role in reducing blood lipids. IN Human Medicinal Agents From Plants
Kinghorn, A.D. & Balandrin, M.F. (Eds) American Chemical Society Books. Washington, D.C. pp1-25.

Lawson, L.D., Wang, Z-Y. & Hughes, B.G. (1991a)
Identification and HPLC quantitation of the sulphides and dialk(en)yl thiosulphinates in commercial garlic products.
Planta Medica 57 pp363-370.

Lawson, L.D., Wang, Z-Y. & Hughes, B.G. (1991b)
γ-Glutamyl-S-alkylcysteines in garlic and other *Allium spp.*: precursors of age-dependant *trans*-1-propenyl thiosulphiates.
Journal of Natural Products 54 pp436-444.

Lawson, L.D., Wood, S.G. & Hughes, B.G. (1991c)
HPLC analysis of allicin and other thiosulphinates in garlic clove homogenates.
Planta Medica 57 pp263-270.

Lawson, L. & Hughes, B.G. (1992)
Characterization of the formation of allicin and other thiosulphinates from garlic.
Planta Medica 58 pp345-350.

Leach, W.D., Lee, A. & Stubbs, R.P. (1973)
Localization of bacteria in the gastrointestinal tract: a possible explanation of intestinal spirochaetosis.
Infection and Immunity 7 (6) pp961-972.

Lehman, F.A. (1930)
Pharmacology of *Allium sativum*.
Archives of Experimental Pathology Pharmakology 147 pp245-264.

Levine, S.M., Gefland, M., Hersh, T., Wyshak, G., Spiro, H.M. & Floch, M.H. (1970)
Intestinal bacterial flora after total and partial colon resection.
Digestion and Disease 15 pp523-528.

Luckey, T.D. (1972)
Introduction to intestinal microecology.
American Journal of Clinical Nutrition 25 pp1292-1294.

Luckey, T.D. (1974)
Introduction: the villus in chemostat man.
The American Journal of Clinical Nutrition 27 pp1266-1276.

Macfarlane, G.T., Cummings, J.H. & Allison, C. (1986)
Protein degradation by human intestinal bacteria.
Journal of General Microbiolgy 132 pp1647-1656.

Macfarlane, G.T., Gibson, G.R. & Cummings, J.H. (1992)
Comparison of fermentation reactions in different regions of the human colon.
Journal of Applied Bacteriology 72 pp57-64.

Maffei, H.V.L. & Nobrega, F.J. (1975)
Gastric pH and microflora of normal and diarrhoeic infants.
Gut 16 pp719-726.

Mallory, A., Savage, D.C., Kern, F. & Smith, J.G.G. (1973)
Patterns of bile acids and microflora in the human small intestine, II. Microflora
Gastroenterology 64 (1) pp34-42.

Manning, W.J. & Moore, G.S. (1977)
Effects of an aqueous garlic extract on several plant pathogenic fungi.
Proceedings of the American Phytopathological Society 4 pp192

Mantis, A.J. Karaioannoglou, P.G., Spanos, G.P. & Panetsos, A.G. (1978)
The effect of garlic extract on food poisoning bacteria in culture media. I. *Staphylococcus aureus.*
Lebensm Wiss U-Technol 11 pp26-28.

Mantis, A.J., Koidis, P.A., Karaioannoglou, P.G. & Panetsos, A.G. (1979)
Effect of garlic extract on food poisoning bacteria.
Lebensm Wiss U-Technol 12 pp330-332.

Maslin, D.J. & Hill, D.J. (1990)
The effects of essential garlic oil (EGO) upon human enteric bacteria.
Industrial Report to Seven Seas Ltd.

Mazelis, M. & Crews, L. (1968)
Purification of the alliin-lyase of garlic, *Allium sativum L.*
Journal of Biochemistry 108 pp725-730.

Meynell, G.G., & Meynell, E. (1970)
Theory and practice in Experimental Bacteriology.
Cambridge University Press. Cambridge. UK.

Middlebrook, J.L. & Dorland, R.B. (1984)
Bacterial toxins: cellular mechanisms of action.
Microbiological Reviews 48 pp199-221.

Minami, T., Boku, T., Inada, K., Morita, M. & Okazaki, Y. (1989)
Odour components of human breath after ingestion of grated raw garlic.
Journal of Food Science 54 pp763-765.

Minekus, M., Marteau, P., Havenaar, R. & Huis in't Veld, J.H.J. (1993)
Development of a computer controlled *in vitro* model of the gastrointestinal tract.
Gastroenterology 104 A553.

Mitsuoka, T. (1992)
The human gastrointestinal tract. IN The Lactic Actid Bacteria Volume I.
Wood, B.J.B. (Ed) Elsevier Applied Science, London, England. pp 69-114.

Mochizuki, E., Nakayama, A., Kitada, Y., Saito, K., Nakazawa, H., Suzuki, S. & Fujita, M. (1988)
Liquid chromatographic determinations of alliin in garlic and garlic products.
Journal of Chromatography 455 pp271-277.

Montet, J.C., Lindheimer, M., Gerolami, A., Montet, A.M., Reynier, M.O., Crotte, C. & Brun, B. (1983)
Intestinal cholesterol uptake from mixed micelles. Effects of micellar size and detergent capacity. IN Bile Acids & Cholesterol In Health & Disease-FALK Symposium
Paumgarter, G., Stiehl, A. & Gerok, W. (Eds) MTP Press Ltd, England. pp223-233.

Molly, K., Vande Woestyne, M. & Verstraete, W. (1993)
Development of a 5-step multi-chamber reactor as a simulation of the human intestinal microbial ecosystem.
Applied Microbiology Biotechnology 39 pp254-258.

Moore, W.E.C., & Holdeman, L.V. (1974)
Human faecal flora: the normal flora of 20 Japaneses-Hawaiians.
Applied Microbiolgy 27 pp961-979.

Müller, B. (1989)
Analytische Bewertung von Knoblauchpräparaten. (English Abstract).
Deutsche Apotheker Zeitung 46 pp2500-2504.

Nagai, K. (1973)
Experimental studies on the preventive effect of garlic extract against infection with influenza virus.
Journal of Japanese Association of Infectious Disease 47 pp321-325.

Nelson, D.P. & Mata, L.J. (1970)
Bacterial flora associated with the human gastrointestinal mucosa.
Gastroenterology 58 (1) pp56-61.

Newton, L., Hall, S.H., Pelerin, M. & McLauchlin, J. (1990)
Listeriosis Survellance.
Communicable Disease Report (1991) 1 R110-3.

Nishino, H., Iwashima, A., Itakura, Y., Matsuura, H. & Fuwa, T. (1989)
Antitumor promoting activity of garlic extracts.
Oncology 46 pp277-280.

Norrby, R. (1984)
Quality of antibiotic clinical trials.
Journal of Antimicrobial Chemotherapy 2 pp1-3.

Onderdonk, A.B., Johnston, J., Mayhew, J.W. & Gorbach, S.L. (1976)
Effect of dissolved oxygen and Eh on *Bacteroides fragilis* during continuous culture.
Applied and Environmental Microbiology 31 (2) pp168-172.

Pastuer, L. (1858)
Memoire sur la fermentation appelee lactique.
Mem Soc Imp Sci Agr Art Lille Ser 2 (5) pp13-

Pentz, R., Guo, Z. & Siegers, C.P. (1991)
Bioactivity and metabolism of thio compounds from different garlic preparations.
Second International Garlic Symposium: Pharmacy, Pharmacology and Clinical Application of *Allium sativum.* Berlin 7-10 March. pp14.

Petricic, J. & Lulic, B. (1977)
Antimicrobial efficiencies and stabilities of active components of garlic (*Allium sativum*).
Acta. Pharm. Jugoslav 27 pp35-40.

Petricic, J. & Lulic, B. (1978)
Garlic (*Allium sativum L*), antifungal effect of some components of volatile oil.
Acta. Pharm. Jugoslav 28 pp41-43.

Plaut, A.G., Gorbach, S.L., Nahas, L. & Weinstein, L. (1967)
Studies of intestinal microflora III. The microbial flora of human small intestinal mucosa and fluids.
Gastroenterology 53 (6) pp868-873.

Porter, J.R. & Rettger, L.F. (1940)
Influence of diet on the distribution of bacteria in the stomach, small intestine and cecum of the white rat.
Journal of Infectious Diseases 66 pp104-110.

Qian, Y.X., Shan, P.J., Xu, R.Y., Liu, G.H., Yang, H.Q., Lu, Y.S., Sun, P., Zhang, R.W., Qi, L.H. & Lu, Q.H. (1986)
Spermicidal effect *in vitro* by the active principle of garlic.
Contraception 34 pp295-302.

Rao, R.R., Rao, S.S. & Venkataraman, P.R. (1946)
Investigations on plant antibiotics. Part 1- studies on allicin, the antibacterial principle of *Allium sativum* (garlic).
Journal of Scientific & Industrial Research vol 1B (12) pp31-35.

Rees, L.P., Minney, S.F., Plummer, N.T., Slater, J.H. & Skyrme, D.A. (1993)
A quantitative assessment of the antimicrobial activity of garlic (*Allium sativum*).
World Journal of Microbiology and Biotechnology 9 pp303-307.

Robson, J.E. & Stockley, H.M. (1962)
Sulphydryl metabolism of fungi grown in submerged culture.
Journal of General Microbiology 28 pp57-68.

Rosenberg, M., Perry, A., Bayer, E.A., Gutnick, D.L., Rosenberg, E. & Ofek, J. (1981)
Adherence of *Acinetobacter calcoaceticus* RAG-1 to human epithelial cells and to hexadecane.
Infection & Immunology 33 pp29-33.

Rosin, S., Tuorila, H. & Uutela, A. (1992)
Garlic: a sensory pleasure or a social nuisance?
Appetite 19 pp133-143.

Rotimi, V.O. & Duerden, B.I. (1981)
The development of the bacterial flora in normal neonates.
Journal of Medical microbiology 14 pp51-62.

Russell, A.D. (1974)
Factors influencing the activity of antimicrobial agents: an apprasial.
Microbios 10 pp154-174.

Saghir, A.R., Mann, L.K., Bernhard, R.A. & Jacobsen, J.V. (1964)
Determination of aliphatic mono- and disulphides in *Allium* by gas chromatography and their distribution in the common food species.
American Society for Horticultural Science 84 pp386-398.

Sanford, P.A. (1992)
Digestive System Physiology: Physiological Principles of Medicine Series.
Edward Arnold. London, UK.

Savage, D.C. (1970)
Associations of indigenous microorganisms with gastrointestinal mucosal epithelia.
American Journal of Clinical Nutrition 23 (11) pp1495-1501.

Savage, D.C. (1972a)
Microbial Pathogenicity in Man and Animals.
Smith, H. & Pearce, J.H. (Eds) Cambridge University Press. Cambridge. pp25-57.

Savage, D.C. (1972b)
Associations and physiological interactions of indigenous microorganisms and gastrointestinal epithelia.
The American Journal of Clinical Nutrition 25 pp1372-1379.

Savage, D.C. (1977)
Microbial ecology of the gastrointestinal tract.
Annual Review of Microbiology 31 pp107-133.

Savage, D.C., Dubos, R. & Schedler, R.W. (1968)
The gastrointestinal epithelium and its autochthonous bacterial flora.
Journal of Experimental Medicine 127 pp67-76.

Scarpino, P.V., Deters, L.E. & Niemeier, R.W. (1969)
Investigations of the types and numbers of aerobic and anaerobic bacteria occuring in intestinal specimens obtained from adult human ileostomy and colostomy subjects.
Bacteriological Proceedings 103 (M231) pp103.

Schaechter, M. & Santomassino, K.A. (1962)
Lysis of Escherichia coli by sulphydryl-binding reagents.
Journal of Bacteriology 84 pp318-325.

Schlyter, J.H., Glass, K.A., Loeffelhola, J., Degnan, A.J. & Luchansky, J.B. (1993)
The effects of diacetate with nitrite, lactate or pediocin on the viability of *Listeria monocytogenes* in turkey slurries.
International Journal of Food Microbiology 19 (4) pp271-281.

Schwimmer, S.P. & Mazelis, M. (1963)
Characterization of alliinase of *Allium cepa* (onion).
Archives of Biochemistry and Biophysics 100 pp66-73.

Sharma, V.D., Sethi, M.S., Kumar, A. & Rarotra, J.R. (1977)
Antibacterial property of *Allium sativum* Linn: *in vivo* and *in vitro* studies.
Indian Journal of Experimental Biology 15 pp466-468.

Shashikanth, K.N., Basappa, S.C. & Sreenivasamurthy, V. (1981)
Stimulatory factors of garlic.
Indian Journal of Biochemistry and Biophysics 18 pp79-80.

Shashikanth, K.N., Basappa, S.C. & Sreenivasamurthy, V. (1984)
A comparative study of raw garlic extract and tetracycline on caecal microflora and serum proteins in albino rats.
Folia Microbiology 29 pp348-352.

Shedlofsky, S. & Freter, R. (1974)
Synergisms between ecologic and immunologic control mechanisms of the intestinal flora.
Journal of Infectious Diseases 129 (3) pp296-303.

Semmler, F.W. (1892)
Über das atherische Ol des Knoblauchs.
Archives of Pharmazie 230 pp434.

Skinner, F.A. & Carr, J.A. (1974)
The Normal Microbial Flora of Man
Academic Press. New York.

Skirrow, M.B. (1990)
Foodborne illness - *Campylobacter.*
The Lancet Oct 13 pp921-923.

Small, L.D., Bailey, J.H. & Cavallito, C.J. (1947)
Alkyl thiosulphinates.
Journal of the American Chemical Society 69 pp1710-1713.

Smalley, E.B. & Hansen, H.N. (1962)
Penicillium decay of garlic.
Phytopathology 52 pp666-678.

Smith, H.W. (1966)
The antimicrobial activity of the stomach contents of suckling rabbits.
Journal of Pathology and Bacteriology 91 pp1-9.

Sockett, P.N. & Roberts, J.A. (1991)
The social and economic impact of Salmonellosis. A report of a national survey in England and Wales of laboratory confirmed *Salmonella* infections.
Epidemiology and Infection 107 pp335-347.

Sockett, P.N., Cowden, J.M., LeBaigue, S., Ross, D., Adak, G.K. & Evans, H. (1993)
Foodborne disease surveillance in England and Wales: 1989-1991.
Communicable Disease Report Review 3 (12) R159-R173.

Sreenivasamurthy, V., Sreekantiah, K.R. & Johar, V.S. (1961)
Studies on the stability of allicin and alliin present in garlic.
Journal of Scientific & Industrial Research 20 (c) pp292-295.

Stark, P.L. & Lee, A. (1982)
The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life.
Journal of Medical Microbiology 15 pp189-203.

Stoll, A. & Seebeck, E. (1948)
Allium compounds I. Alliin the true mother compound of garlic oil.
Helvetica Chimica Acta 31 pp189-210.

Stoll, A. & Seebeck, E. (1951)
Chemical investigations on alliin, the specific principle of garlic.
Advanced Enzymology 11 pp377-400.

Subrahmanyan, V., Krishnamurthy, K., Sreenivasamurthy, V. & Swaminathan, M. (1957a)
Effect of garlic in the diet on the intestinal microflora of rats.
Journal of Scientific and Industrial Research 16C (9) pp173-174.

Subrahmanyan, V., Sreenivasamurthy, V., Krishnamurthy, K. & Swaminathan, M. (1957b)
Studies on the antibacterial activity of spices.
Journal of Scientific and Industrial Research 16C (9) pp240-241.

Subrahmanyan, V., Krishnamurthy, K., Sreenivasamurthy, V. & Swaminathan, M. (1958)
The effect of garlic on certain intestinal bacteria.
Food Science 7 pp223-224.

Tabaqchali, S., Okubadejo, O.A., Neale, G. & Booth, C.C. (1966)
Influence of abnormal bacterial flora on small intestinal function.
Proceedings of the Royal Society of Medicine 59 pp1244-1246.

Tally, F.P (1993)
Bacteroides and Abscesses. IN Mechanisms of Microbial Disease
Schaechter, M., Medoff, G. & Eisenstein, B.I. (Eds) Willams & Wikins. London. pp244-250.

Tannock, G.W. (1983)
Effect of dietary and environmental stress on the gastrointestinal microbiota. IN Human Intestinal Microflora In Health and Disease
Hengtes, D.J. (Ed) Academic Press. New York. pp517-539.

Tannock, G.W. & Savage, D.C. (1974)
Influences of dietary and environmental stress on microbial populations in the murine gastrointestinal tract.
Infection and Immunity 9 (3) pp591-598.

Tansey, M.R. & Appleton, J.A. (1975)
Inhibition of fungal growth by garlic extract.
Mycologia 67 pp400-413.

Thadepalli, H., Lou, M.A., Bach, V.T., Matsui, T.K. & Mandal, A.K. (1979)
Microflora of the human small intestine.
American Journal of Surgery 138 pp845-850.

Tsai, Y., Cole, L.L., David, L.E., Lockwood, S.J., Simmons, V. & Wild, G.C. (1985)
Antiviral properties of garlic: *In vitro* effects on influenzae B, herpes simplex and Coxsackie viruses.
Planta Medica 5 pp460-461.

Tynecka, Z. & Gos, Z. (1973)
The inhibitory action of garlic (*Allium sativum L.*) on growth and respiration of some microorganisms.
Acta Microbiologica Polonica SerB. 5 (22) 1 pp51-62.

Vandenbergh, P.A. (1993)
Lactic acid bacteria, their metabolic products and interference with microbial growth.
Federation of European Microbiological Societies Reviews 12 (1-3) pp221-238.

Vercelotti, J.R., Salyers, A.A., Bullard, W.S. & Wilkins, T.D. (1977)
Breakdown of mucin and plant polysaccharides in the human colon.
Canadian Journal of Biochemistry 55 pp1150-1196.

Vernin, G., Metger, J., Fraisse, D. & Scharff, C. (1986)
GC-MS (EI, PCI, NCI) computer analysis of volatile sulphur compounds in garlic essential oils. Application of the mass fragmentometry SIM technique.
Planta Medica 52 pp96-101.

Walton, L., Herbold, M. & Lindegren, C.C. (1936)
Bactericidal effects of vapours from crushed garlic.
Journal of Agricultural Research 41 pp163-169.

Weber, N.D., Anderson, D.O., North, J.A., Murray, B.K., Lawson, L.D. & Hughes, B.G. (1992)
In vitro virucidal effects of *Allium sativum* (garlic) extract and compounds.
Planta Medica 58 pp417-423.

Willis, A.T., Bullen, C.L., Williams, K., Fagg, C.G., Bourne, A. & Vignon, M. (1973)
Breast milk substitute: A bacteriologic study.
British Medical Journal 4 pp67-72.

Wills, E.D. (1956)
Enzyme inhibition by allicin, the active principle of garlic.
Journal of Biochemistry 63 pp514-520.

Wolfe, M.M. & Soll, A.H. (1988)
The physiology of gastric acid secretion.
The New England Journal of Medicine 319 (26) pp1707-1715.

MISSING

PAGES

NOT

AVAILABLE

World Cancer Research Fund (1990)
Healthy eating.
Newsletter June 3.

World Health Organization Working Group (1988)
Foodborne Listeriosis.
Bull. World Health Organization 66 pp421-428.

Yan, X., Wang, Z. & Barlow, P. (1992)
Quantitative estimation of garlic oil content in garlic oil based health products.
Food Chemistry 45 pp135-139.

Yan, X., Wang, Z. & Barlow, P. (1993)
Quantitative determination and profiling of total sulphur compounds in garlic health products using a simple GC procedure.
Food Chemistry 47 pp289-294.

Yu, T.H. & Wu, C.H. (1989)
Stability of allicin in garlic juice.
Journal of Food Science 54 (4) pp977-981.

Yue, Z., Sun, Y., Liu, X., Liao, J., Yao, S., Li, Y., Chen, X. & Fang, L. (1984)
Effect of allitridi on platelet aggregation, a preliminary study.
Journal of Traditional Chinese Medicine 4 pp29-32.

Ziegler, S.J. & Sticher, O. (1989)
HPLC of S-alk(en)yl-L-cysteine derivatives in garlic including quantitative determination of (+)-S-allyl-L-cysteine sulfoxide (alliin).
Planta Medica 55 pp372-378.

Zubrzycki, L. & Spaulding, E.H. (1962)
Studies on the stability of the normal human fecal flora.
Journal of Bacteriology 83 pp968-974.